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THE WERNER K. NOELL

Friedenwald Memorial

PROCEEDINGS

of the

Association for Research in Ophthalmology, Inc.

Twenty-eighth Annual Meeting

Atlantic City, New Jersey

June 9, 10 and 11, 1959

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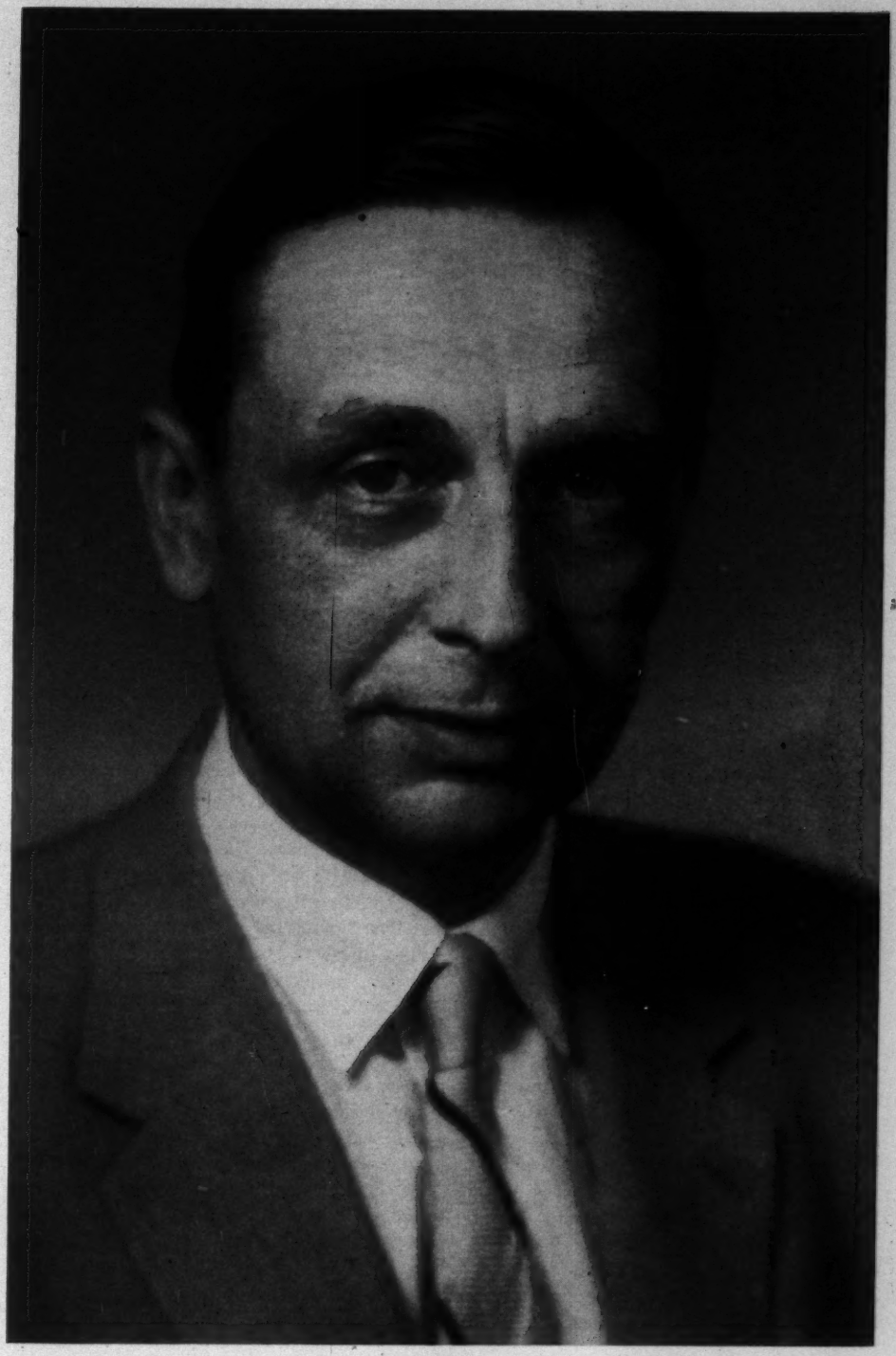

WERNER K. NOELL, M.D.

REMARKS ON ACCEPTANCE OF FRIEDENWALD MEMORIAL PLAQUE

WERNER K. NOELL, M.D.
Buffalo, New York

I am very thankful for the honor and privilege of presenting the Jonas Friedenwald Lecture. I do so under the assumption that the honor has been awarded really to the many investigators who, like myself, have elected retinology as their most cherished subject of scientific endeavor. In their behalf I want to thank the association for providing a forum, indeed the only existing one, for retinologists of all kinds to meet and exchange ideas, and to learn from their clinical colleagues and from ophthalmologic scientists in general. In my own behalf I wish to thank the association for the encouragement I have received from within its ranks.

I am deeply moved to have been chosen to participate in the tribute to Dr. Friedenwald. To me Dr. Friedenwald is the symbol of a man who is inspiring by his human qualities no less than by his exceptional intellectual and professional accomplishments. The spirit in which this association honors his memory is testimony to his greatness and to the affection and respect of his colleagues, old and young alike. Speaking as a researcher, I admire his courage to extend his activities into fields beyond the boundaries of his immediate interest and I wish I had his ability to be proficient in so many areas of investigation.

Dr. Friedenwald's name is well known and respected outside of ophthalmology. As a matter of fact, few cytologists, histochemists, or physiologists I have met are aware of the fact that he was an ophthalmologist rather than one of their own breed. His diverse researches and interests enabled him to look at a problem from many viewpoints and to integrate information from various specialized fields. Such men have been the leaders of the scientific community at all times but the need for integrating leadership throughout biology and medicine is felt more urgently today than ever before because the techniques have been pushed to a phenomenal level of specialization.

The danger that we become embroiled in the narrow aspects of our techniques and lose sight of our common goal is only too apparent. I think that contact between the various disciplines and between researchers and clinicians as fostered by the Association for Research in Ophthalmology is the strongest force presently preventing this from happening. The association is indeed fortunate to have been under the influence of Dr. Friedenwald's leadership and to be endowed with his spirit for integration of the clinical and biologic aspects of ophthalmologic problems.

BIOGRAPHY: WERNER K. NOELL, M.D.

I was born in Germany in 1913, the son of a general practitioner in a small town. I was destined to study medicine and in due course was graduated from the University of Hamburg (1937). My particular interest during the medical school years was neurology and most of my internship year as well as a postinternship period was spent at the Neurological Clinic, University of Hamburg. Planning to return after two or three years to clinical work I began, in 1939, postgraduate studies in physiology at the University of Berlin. I soon was engaged in research on cerebral circulation and oxygen consumption, most of which was conducted together with my teacher in this field, Max Schneider, at the Medical School in Danzig to which we moved in 1940.

The need to supplement our circulatory determinations with a measure of brain func-

tion brought me, in 1943, to the Max-Planck Institut Für Hirnforschung (brain research) then in Berlin-Buch where A. E. Kornmüller had established a leading laboratory of electroencephalography. I was by then a "Privat-Dozent" in Physiology at the University of Cologne but, because of the war, I stayed at the Brain Research Institute until 1946 when as a result of my interests in brain hypoxia, I had the opportunity to come to the School of Aviation Medicine at Randolph Field, Texas, and to continue my studies there for a little while. I was associated with the Department of Neuropsychiatry and headed the section of Neurology and Electroencephalography. Again contrary to plans, I stayed much longer, enjoying the remarkable research facilities. It was at Randolph Field that I became interested in retinal physiology.

Late in 1954 I moved to the Roswell Park Memorial Institute in Buffalo to be a principal research scientist and the head of the neurophysiological laboratory in the Department of Anesthesiology. I am an associate research professor in physiology of the Graduate School of Arts and Sciences, University of Buffalo, Roswell Park Division. I teach medical students on the campus and graduate students in my laboratory, where I have been giving a course in brain electrophysiology. I participate in clinical electroretinography with A. D. Ruedemann, Jr., at the Kresge Eye Institute, Detroit. My major field of interest is the physiology of retina and brain with respect to relationships between function and metabolism.

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THE VISUAL CELL: ELECTRIC AND METABOLIC MANIFESTATIONS OF ITS LIFE PROCESSES*

THE FRIEDENWALD MEMORIAL LECTURE

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Throughout the period of modern biology, the retina, because of its remarkably ordered structure and unusual function, has attracted the attention of both morphologists and physiologists. This has been especially true of the visual cell. If there were need to demonstrate that a highly specialized function requires a highly specialized structure, then the visual cell would illustrate this in a very beautiful manner. According to the contemporary view, function, whatever it may be, is the consequence of metabolic processes and we may expect that the metabolism of the visual cell is specialized in several respects to suit the particular function of the cell.

In this lecture, I would like to present some factual information on these three aspects of visual cell biology: the metabolism, structure, and function of the cell, and I shall try when possible to relate these to each other. I shall be guided mainly by our own researches in which the principal approach has been to study the relationship between metabolism and function by determining the effects of various kinds of metabolic interferences on the visual cell, particularly on the electrical manifestations of its activity.[1-6] An appropriate place to begin is with our studies[7-10] of the metabolism of the retina

* From the Roswell Park Memorial Institute.

since they provide the biochemical background for further discussion.

In vitro metabolism of rabbit retina

Since the visual cells are a major part of the retina, the study of the total metabolism of this tissue is a source of information on the metabolic activities of the visual cell. Virtually nothing is known of retinal metabolism in vivo and even the literature on the in vitro activities of the retina is scant and not as informative as needed for the consideration of relationships between function and metabolism. We, therefore, decided to study how the retina of the rabbit, our main experimental animal, behaves in vitro. We have concentrated upon the metabolism of glucose partly because glucose must be the primary source of energy for retinal function and partly because the retina of the rat and some other species is long known to produce lactic acid in vitro at exceptionally high rate.[11-16]

Figure 1 presents in simplified form the presently known paths by which glucose is catabolized in animal tissues.[17] In the Embden-Meyerhof pathway glucose after phosphorylation at the sixth carbon atom is cleaved and the fragments oxidized to yield two molecules of pyruvic acid. We can visualize two kinds of pyruvate molecules, those coming from the first three carbon atoms of glucose and those derived from the lower three. The pyruvate so formed will be either reduced to lactate or, if oxygen is available, oxidized to carbon dioxide and water by way of the citric acid cycle. If oxidation occurs, pyruvate is degraded by a series of stepwise oxidation in which its carbon atoms are converted to CO_2 one at a time. A brief description of this process is necessary for the understanding of some of our results obtained in experiments with radioactive glucose.

As indicated in Figure 1, the first molecule of the CO_2 formed from pyruvate contains the carbon which was originally in position 3 or 4 of the glucose molecules depending upon whether the pyruvate came from the first half (1-2-3) or the second half (4-5-6) of glucose; the next molecule of CO_2 contains the carbon from position 2 or 5, and the third from position 1 or 6. Actually, the second and third molecule of CO_2 arise during successive turns of the acetyl fragment in the citric acid cycle, carbon 2 or 5 released as CO_2 during the second turn and carbon 1 or 6 during the third turn.

The fate of the various carbon atoms of glucose can be traced by the use of glucose containing radioactive carbon-14 either in a

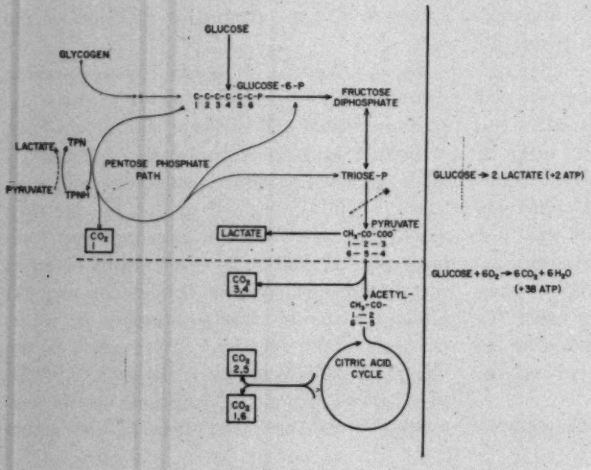


Fig. 1 (Noell). Pathways of glucose metabolism. The heavy arrows trace the Embden-Meyerhof pathway and the citric acid cycle, the asterisk indicates the main site of iodoacetate inhibition (triose-phosphate dehydrogenase). The boxes denote endproducts. Reactions below the dashed horizontal line require oxygen.

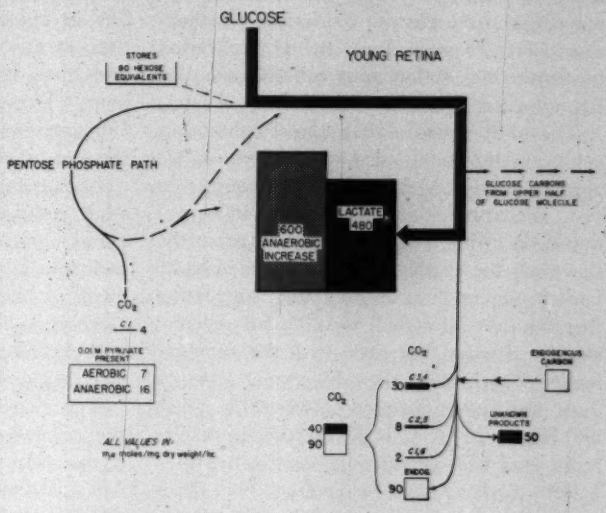


Fig. 2 (Noell). Glucose metabolism of retinas from five to seven-day-old rabbits as measured in Krebs-Ringer-phosphate buffer. All data from references 9 and 10. Glucose concentration: 0.02 M. The height of each block (and the number inside or beside each block) represents the millimicromoles of lactic acid or CO_2 produced per mg. dry weight per hour; the area of each block represents the millimicro*atoms* of glucose *carbon* (black) or of *carbon* from endogenous substrate (white) released as CO_2 or accumulated as lactate. The conversion of the various glucose carbon atoms to CO_2 was estimated from measurements in which the glucose of the medium was labelled by carbon-14 in position 1, 2, 6 or uniformly in all six positions.[10] Anaerobic lactic acid production is the sum of the two blocks, one marked "lactate," the other marked "anaerobic increase."

single carbon position only, or in all six positions uniformly. Replicate experiments performed under the same conditions but with the glucose of the medium labelled differently furnish information on the proportion of the glucose which is completely oxidized, and indicate at which steps glucose fragments escape further oxidation and accumulate. These experiments, moreover, enable one to determine how much of the carbon dioxide or lactate produced by the tissue is formed from the glucose of the medium and how much from endogenous substrates, that is, substrates originally present in the tissue.

In the hope of better tracing relationships between function and metabolism, we compared the adult, fully developed retina with the retina of the five to seven-day-old rabbit.[6-8] At this age the retina is still not functioning and the rods have not yet grown much beyond small buds.

Figure 2 summarizes in a schematic form some of our results with the young retina. The height of each block represents the amounts of lactic acid or carbon dioxide produced on a molar basis and the area represents the total carbons there collected; black areas indicate carbons coming from the glucose of the medium, light areas those from endogenous sources.

The most remarkable property of the young retina is its high lactic acid production under aerobic condition, all of the lactic acid coming from the glucose of the medium. By comparison, very little glucose is degraded by the oxidation of pyruvate as indicated by the small amount of glucose carbon appearing as CO_2. Moreover, of the pyruvate molecules entering the oxidative route, few are completely oxidized. This is shown by the large difference between the carbon dioxide originating from glucose carbon 3 and 4 and that coming from glucose carbon 1 and 6. Indeed more glucose carbon (=50) accumulates as metabolic intermediates via the citric acid cycle (marked unknown products in Figure 2) than are converted to carbon dioxide (=40). There is, however, a considerable oxidation of endogenous substrate and, in fact, about twice as much respiratory carbon dioxide is formed from endogenous sources than is formed from glucose.

In short, the young retina is equipped with a metabolic machinery capable of producing

lactic acid at a very high rate compared to respiration and glucose oxidation. Furthermore, for its respiration the young retina prefers to use endogenous substrates rather than glucose.

The adult retina in vitro (fig. 3) shows an even greater lactic acid production under aerobic condition than does the young; in fact, almost twice as much lactic acid is produced on a dry weight basis. Anaerobically, however, the lactic acid formed is only 25 percent higher than in the young, suggesting that the over-all retinal capacity for glycolysis has changed but little with the development of the retina to a functional state. A very striking difference between the adult and immature retina is the increase in respiration (see CO_2 production) and in the utilization of glucose for respiration. Five times more pyruvate from the glucose of the medium enters the oxidative path than in the young and a much greater fraction of this pyruvate is completely oxidized in the citric acid cycle. The contribution of endogenous carbon sources to respiration has changed little, and most of the respiration, almost 75 percent, is accounted for by the oxidation of glucose. All these changes mainly occur between the age of 11 days and 20 days when outer and inner limbs become adult-like in appearance and when retinal function, as measured by the electroretinogram, undergoes its most rapid phase of development. However, complete maturation in function and structure is not attained until the age of about three to four months.

In summary, the in vitro respiration of the adult retina of the rabbit is one of preferential glucose oxidation, this property making its appearance during the development of the retina to a functioning state. Aerobic lactic acid production is very high and exceeds oxygen consumption about two-fold on a molar basis.

The characteristics of the metabolism of the isolated retina are best appreciated by a comparison with other tissues measured under the same conditions. Calculations from the data of McIlwain[19] indicate that cerebral cortex slices (gray matter) respire at only about half the rate of the adult retina, while their lactic acid production is aerobically at best 15 percent and anaerobically about 35 percent that of the adult retina. Oxygen consumption exceeds aerobic lactic acid production by a factor of two while the reverse is true for the retina. No data for comparison are available on the glucose oxidation of

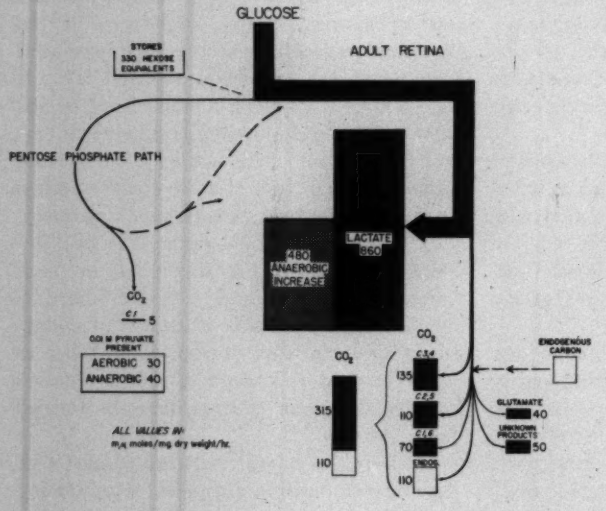


Fig. 3 (Noell). Glucose metabolism of retinas from adult rabbits measured under the same conditions as the young retina. All data from references 9 and 10. Results are presented in the same manner as in Figure 2.

brain slices but for the in vivo condition it is known that the direct oxidation of glucose is only about one-third of brain respiration, the larger part of respiration being due to the oxidation of endogenous non-carbohydrate substrates which are resynthesized from glucose fragments.[20] In comparison with cancer tissue, the retinal metabolic rates are also generally high. For example, per unit dry weight the adult retina surpasses in both respiration and lactic acid production ascites tumor cells (Ehrlich-Lettré); however, the initial rate of lactic acid production is higher in tumor cells than in the retina.[21-22]

Warburg[23] assumed that the high aerobic lactate production of the retina—unusual for a normal tissue but typical for cancer—were the result of damage arising from the separation of the retina from the eye. The point cannot be settled until in vivo data are available. However, from calculations of the diffusion of oxygen through the rabbit's retina[24] and from polarographic measurements of the oxygen tension at the inner surface,[25] it appears almost impossible that the retina in vivo respires at the same high rates measured in vitro. If oxygen from the blood cannot diffuse fast enough to satisfy respiratory rates as high as those observed in vitro, one might doubt that the elimination of lactic acid would proceed in vivo rapidly enough to maintain a physiological pH in the face of a lactic acid production as high as that measured in vitro. We rather assume that retinal metabolism is restrained in vivo, and the ready loss of this restraining control might be a characteristic property of the retina.

The isolated rabbit's retina does not lack controls which other tissues generally display; for instance, it responds as well as other tissues to agents which uncouple phosphorylation from respiration and, like brain slices, the respiration of the adult retina is markedly accelerated by the omission of calcium ions from the medium. The adult retina of the rabbit differs from other tissues (and from the young retina), however, in the apparently low degree of control of glycolysis by respiration, as shown by the relatively small difference between aerobic and anaerobic lactic acid production. We assume this to suggest that the adult retina is heterogenous in its metabolic organization, and that the sites of high glycolytic capacity are in part separated from sites of high respiratory capacity.

What now is the contribution of the visual cells to the over-all in vitro metabolism of the retina? The answer to this question is in part given by measurements of Lowry, et al.[26] on the distribution of enzymes of glycolysis and respiration over the retinal layers. From these measurements and my own data[6] on retinas lacking visual cells (due to iodoacetate poisoning as later discussed) it seems reasonable to assume that half of the in vitro respiration and half of the aerobic lactic acid production of the adult rabbit's retina is contributed by the visual cells, whereas anaerobically about two-thirds of the lactic acid comes from the inner layers. The rabbit's retina has no blood capillaries throughout its layers and all oxygen must diffuse through the retina from the choroid. This property probably explains why the cells of the inner layers, as shown by Lowry, et al.,[26] are relatively high in glycolytic enzymes and relatively low in enzymes of the citric acid cycle. In the monkey, Lowry, et al., find that the enzyme pattern of the inner layers is the opposite of that in the rabbit while the pattern for the visual cell is very similar. Possibly, therefore, the high rate of lactic acid production by retinas of species where the inner layers are vascularized may reflect principally visual cell activity under aerobic conditions. My study[27] of retinas from mice lacking the visual cells due to hereditary degeneration indeed suggest this to be the case.

Retinal research is as well plagued as benefited by many species differences and one further species-dependent property should here be mentioned. As outlined earlier, the respiration of the adult rabbit retina is

mainly supported by glucose oxidation, the oxidation of endogenous substrates being very low. In contrast the respiration of the ox retina in the same medium as used in our studies appears to be supported to a very large extent by endogenous substrates.[28-29]

Lowry's enzyme measurements[26, 30-31] have brought forth the important finding that the initial enzymes of the second main route of glucose catabolism—the pentose phosphate pathway—are present in exceptionally high concentration in the outer synaptic layer and the inner limb. While the glycolytic route and the citric acid cycle are the major sources of cell energy (ATP), the main function of the pentose phosphate path is believed to be the production of reduced triphosphopyridine nucleotide (TPNH) for use in synthetic reactions requiring reduction.

As indicated in Figure 1, the operation of the pentose phosphate path gives rise to carbon dioxide from carbon-1 of glucose. By measuring the carbon dioxide formed from glucose carbon-1 in comparison to that formed from other glucose carbon atoms an estimate of the activity of the pentose phosphate path can be achieved. This estimation involves complicated considerations which have been discussed elsewhere.[10] As shown in Figures 2 and 3, the activity of this path was evident in the young as well as in the adult rabbit retina. It will be noted that in the presence of pyruvate, carbon dioxide production by this route is greatly increased, apparently because the reoxidation of TPNH can be coupled to the reduction of pyruvate to lactate (fig. 1) as was first shown with any tissue by Kinoshita.[32, 33] Under anaerobic conditions and in the presence of pyruvate, carbon dioxide is produced by this route at a rate two and one-half times faster in the adult than in the young retina. This suggests that the development of the retina to a functional state may be associated with a marked increase in the capacity of this pathway. Indeed, Schimke[34] recently reported the glucose-6-phosphate dehydrogenase—one of the initial enzymes of this path—increased four-fold during the post-natal development of the retina. The physiologic role of the pentose-phosphate path in the visual cells has still to be assessed but Lowry's measurements strongly suggest it to be important in visual cell functioning.

In order to provide a basis for further discussion I would like to turn now to a brief description of the in vitro effects of iodoacetate. As shown in Table 1, iodoacetate is

TABLE 1

EFFECTS OF IODOACETATE IN VITRO, EXPRESSED IN PERCENT OF CONTROL VALUES

(Glucose oxidation was measured by determining the radioactivity of CO_2 formed from uniformly labeled C^{14}-glucose. Respiration from endogenous substrate was calculated from the difference between total respiration and glucose oxidation. Capacity of the pentose-phosphate path was determined as the difference between the CO_2 formed from C-1 of glucose and the CO_2 formed from C-6 of glucose in a medium containing pyruvate.)

IAA (M)	Anaerobic Lactic Acid	Aerobic Lactic Acid	Glucose Oxidation	Respiration	Respiration from Endogenous Substrates	Capacity of Pentose-phosphate Path
Adult Retina						
1×10^{-6}	85	92	92	100	117	—
5×10^{-6}	58	62	79	95	130	—
1×10^{-4}	33	32	50	75	130	82
2×10^{-4}	12	18	32	59	120	—
1×10^{-3}	<2	5	6	39	111	59*
Young Retina						
2×10^{-3}	7	10	12	90	123	175

* 2×10^{-3} M IAA.

a powerful depressant of retinal lactic acid production and inhibits glucose oxidation almost as effectively. Total respiration is less sensitive than glucose oxidation, and an increase in the oxidation of endogenous substrate compensates in part for the inhibition of glucose oxidation. Since a much smaller fraction of the respiration of the young retina is supplied by glucose oxidation, its respiration is almost unaffected by the poison even when lactic acid production and glucose oxidation have been virtually blocked. The carbon dioxide production by the pentose phosphate pathway in the presence of pyruvate and oxygen is remarkably increased in the young retina and is only slightly reduced in the adult.

Iodoacetate is long known to be a very effective inhibitor of the triose phosphate dehydrogenase of the Embden-Meyerhof pathway.[35-36] The in vitro changes of retinal metabolism caused by iodacetate can be fully explained on this basis. The increase in the oxidation of endogenous substrates and the failure to observe a significant inhibition of the pentose phosphate pathway strongly suggest that neither the citric acid cycle nor the pentose phosphate pathway are significant sites of the action of the poison. The observed increase in carbon dioxide production by the pentose phosphate route in the young retina probably results from a diversion of glucose-6-phosphate into this route when the EmbdenMeyerhof pathway is blocked and when sufficient respiratory ATP is still available for glucose phosphorylation. By similar reasoning, the observed inhibition of the pentose phosphate pathway in the adult probably is related to the fact that both respiration and glycolysis are markedly affected so that the supply of ATP for the formation of glucose-6-phosphate is not sufficient any more to enable the phosphate-pentose pathway to operate at a high rate.

Selective vulnerability of visual (rod) cell

The unusual features of glucose metabolism in the isolated retina are most probably the basis for the high, selective effectiveness of iodoacetate on function and maintenance of this tissue in vivo. The first indication of a selective effect of iodoacetate upon the retina came during a study of the cerebral effects of anoxia in rabbits.[37] Iodoacetate was injected intravenously in order to determine whether a depression of the Embden-Meyerhof pathway would accelerate cerebral failure resulting from a reduction in oxygen pressure. We were recording the electrical activity of the visual cortex in response to rhythmic flashes of light. Surprisingly, these responses vanished two to five minutes after the injection of the poison, although anoxia had not yet been induced. All other electrical activities of the visual cortex, such as the reactions to alerting auditory or somatic sensory stimuli, were unaffected. In the next experiment, electrodes were inserted into the lateral geniculate body. Here, too, the responses to photic stimulation disappeared. The same was the case for the optic tract potentials.

The effect of iodoacetate upon the responses of the visual pathway suggested a blockage of excitation by the poison somewhere within the retina. The electroretinogram proved this to be the case (fig. 4). The failure of optic tract and visual cortex responses was associated with marked electroretinographic changes involving first the b-wave, and later the other components of the rabbit's electroretinogram until finally, about 20 minutes after the injection, all electrical reactions to photic stimulation had vanished.

The complete extinction of the electroretinogram iodoacetate strongly suggested that the first retinal neuron, the visual cell, was one of the sites of action of the poison. Indeed, the visual cells proved to be the sole or main target of the irreversible effect of the poison, which became evident when the dose exceeded that needed for a marked depression of the electroretinogram.[24] Then the recovery from iodoacetate, which ordinarily occurred within a half to three days,

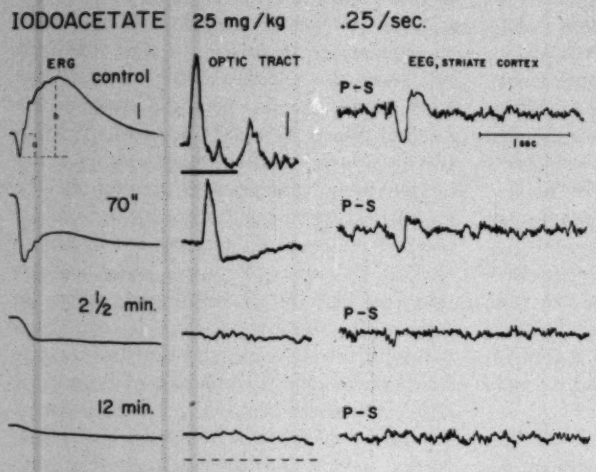


Fig. 4 (Noell). Effect of intravenous iodacetate in rabbit on electroretinogram, optic tract response and electroencephalogram, all measured simultaneously. Electroretinogram and optic tract response were recorded by cathode ray oscilloscope, the sweep was triggered by the on of the flash; flash duration is indicated by heavy line under optic tract tracing. Time base is the same for optic tract response and electroretinogram (note dashed line, each dash represents 10 msec.). The electroencephalogram was recorded on ink, parietal area against striate area (P-S). The deflections in the first third of the electroencephalogram tracing are the responses of the striate area to the light flash. The recording speed of the electroencephalogram is much slower than that of the oscilloscope (note time mark for electroencephalogram labelled one sec.). The light flash is repeated every four seconds ("0.25/sec.").

was incomplete or absent. In inverse relationship to the degree of electroretinogram recovery, visual cell death had occurred. The other cells of the retina survived until much higher doses were employed. Rabbit,[2,4,38–41] cat,[2,4] rat[42] and even the chicken,[43] all show the selective effect of iodoacetate upon the visual cell. Significantly, in the rhesus monkey only the rod cells have this high and selective vulnerability. Iodoacetate was found to differentiate between the rod and cone cells of the rhesus monkey just as does retinitis pigmentosa in man.[2,4] In rabbit and cat, cone cells are also less readily affected than the rod cells, but while this differential effect of iodacetate appears to be a very slight at the light microscopic level, it is of significant magnitude after a low dose at the level of electromicroscopy as recently shown by Lasansky and DeRobertis.[44]

In order to obtain more information on this remarkable vulnerability of retinal function and visual (rod) cell life, we compared by electroretinography the effects of iodoacetate and anoxia in several species of the vertebrate scale.[2] This study strongly suggested that iodoacetate does not produce its retinal effects by interfering with a system which is the exclusive property of the retina as is the rhodopsin system, but rather that it affects retinal function by interfering with general cellular metabolism. For example, iodoacetate at very high dose lacked a consistent effect upon the function of the frog's retina, known for its low aerobic lactic acid production; the combination, however, of iodoacetate poisoning and anoxia eliminated the electroretinogram within two to four minutes, anoxia alone being very slowly effective.

The selective retinal vulnerability to iodoacetate proved to be a particular property of mammals. In these animals, the retinal effect of iodoacetate appeared to surpass that of anoxia especially because certain iodoacetate sensitive components of the electroretinogram were relatively resistant to anoxia. Furthermore, while iodoacetate abolished retinal function without significantly affecting heart and brain, anoxia produced cardiac and cerebral failure while only slowly eliminating the electroretinogram. This is in part

illustrated in Figure 5. As a result of anoxia induced by nitrogen breathing, all electrical activity of the cerebral cortex vanishes in 80 seconds but even then the electroretinogram is still preserved. Moreover, the b-wave (rabbit) which rapidly declines after iodoacetate administration, is not depressed during early stages of anoxia but instead is slightly increased. Cyanide, as shown in Figure 6, produces the same changes. Here, too, the rapid effect upon brain activities and on the optic tract potentials in response to illumination contrasts with the slow effect upon the electroretinogram.

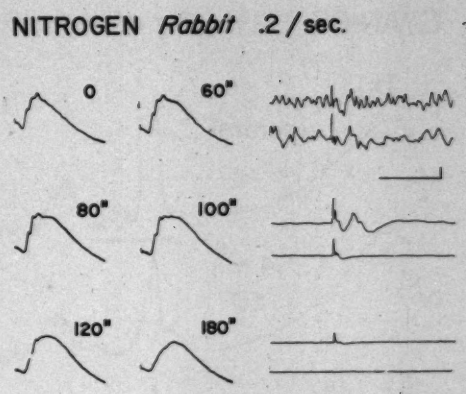


Fig. 5 (Noell). Effect of anoxia on electroretinogram and on electroencephalogram from striate area. Upper electroencephalogram tracing corresponds always to the left electroretinogram of the row, lower electroencephalogram tracing to the right electroretinogram.

Three more points concerning the iodoacetate effect should be stressed. First, while iodoacetate produces the selective death of the visual cells without destroying other retinal cells, no such selective visual cell pathology has been reported ever to result from anoxia; indeed, the literature indicates that on histopathological evidence retinal cells are more resistant to ischemia than are brain cells and that the visual cells resist ischemia better than the cells of the inner layers. Second, while nitrogen breathing or the blockage of blood circulation results in the complete lack of oxygen within a matter of seconds, iodoacetate, at a dose which abolishes the electroretinogram, seems to inhibit simultaneously retinal lactic acid production only 30 to 50 percent as indicated by the in vitro activity of retinas isolated after the injection of iodoacetate, and by the accumulation of lactic acid in the retina of the intact eye separarated from the circulation subsequent to iodoacetate administration.[45-47] Hence, if one compares the effects of anoxia and iodoacetate, one actually compares, from our point of view, complete suppression of respiration with partial inhibition of the Embden-Meyerhof pathway. Third, though one cannot be certain that the effects of iodoacetate upon the electroretinogram are all exclusively the result of visual cell impairment, the complete extinction of the electroretinogram which rapidly follows iodoacetate administration, clearly indicates that visual cell function as well as visual cell life are readily affected by the poison.

These findings, considered together with our knowledge of the action of iodoacetate on triose phosphate dehydrogenase and that retinal aerobic lactic acid production is unusually high, argue very strongly that function and maintenance of the visual cell are extraordinarily dependent upon glycolysis as a process distinct from total cell respiration. It is, of course, possible that glycolysis supplies some metabolic intermediate essential for visual cell excitation. However, the sensitivity to iodoacetate of cell maintenance as well as function suggests that in addition to any specific chemical rôle it may play, glycolysis must be of great general metabolic importance to the visual cell, almost certainly as a source of phosphate bond energy.

In the light of this interpretation we may now consider the possible patterns of the energy supplying systems in the visual cell. It may be instructive to examine three possible extreme cases. In Figure 7, scheme A represents a system where phosphate bond energy is obtained from respiration and where the respiratory machinery is exclusively fed through the glycolytic pathway.

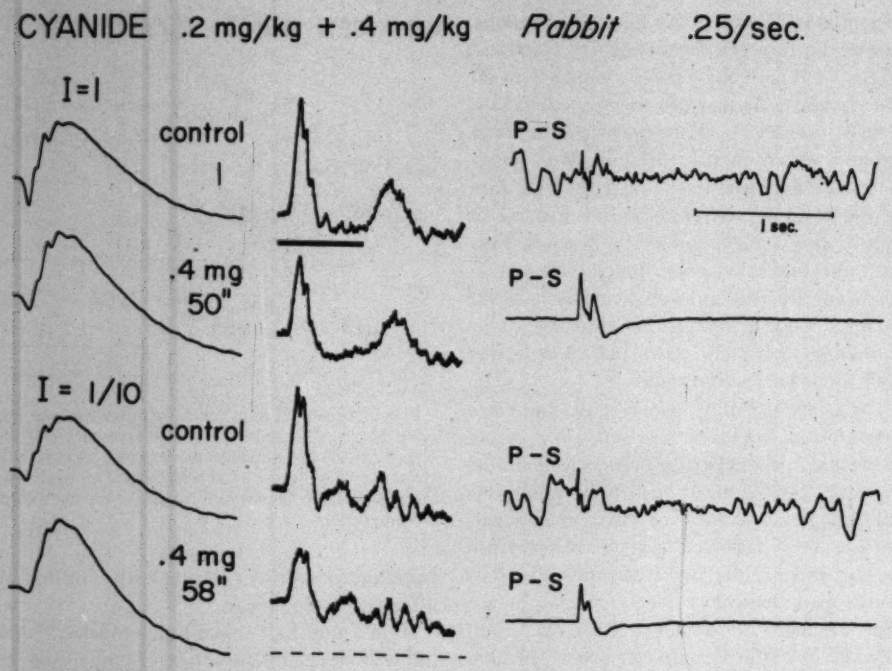


Fig. 6 (Noell). Effect of cyanide on electroretinogram, optic tract response and EEG from striate area. See Figure 4 for explanation. Lower two rows show the responses to a light flash 1/10 the intensity of that used in upper two rows.

B represents a system where the energy is produced by respiration but where the immediate substrate of respiration comes from a noncarbohydrate endogenous store which is replenished from the products of glucose metabolism. C is a system in which all of the available energy arises from glycolysis itself. Since the energy yield from glycolysis is small, considerable amounts of lactic acid must be produced to approach the energy yield of the respiratory system and the retina is certainly equipped with this faculty. Consider now the effects of anoxia: the energy yield from A or B will virtually cease since they are primarily dependent upon respiration, while system C will be unaffected. Conversely, iodoacetate due to its inhibitory effect upon glycolysis, will eliminate energy production by C, will leave B unaffected until the endogenous store becomes depleted and will eliminate system A by cutting off the supply of respiratory substrate.

In view of our finding that glucose is the principal source of respiratory carbon dioxide in the adult retina, scheme B may be discarded, and evidently then anoxia would not be as effective as complete inhibition of glycolysis. However, the high effectiveness of a partial inhibition of the Embden-Meyerhof pathway compared to that of anoxia favors the assumption that a system like C, in which the available energy is principally obtained from glycolysis, is of very special importance for visual cell function and cell life. When this conclusion was first made, the fine structure of the visual cell was still unknown, but today the concept is supported by electromicroscopic evidence.

Figure 8 gives a schematic presentation of the electronmicroscopic appearance of the rabbit's rod cell as described by De Robertis[48] and De Robertis and Franchi.[49] The

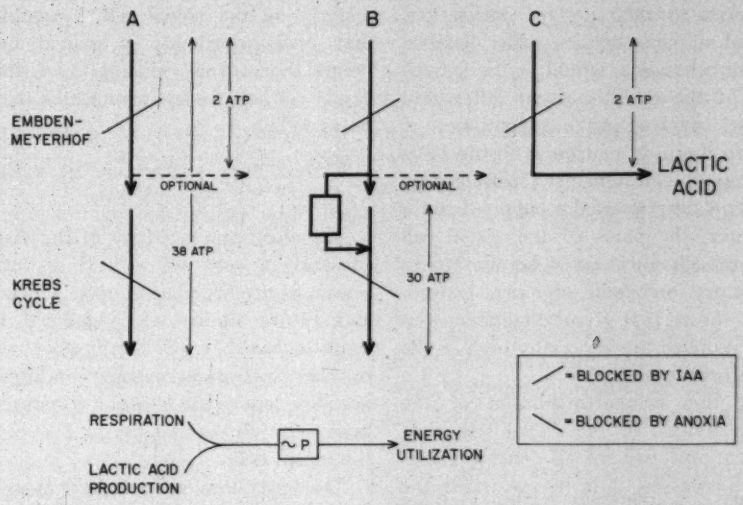
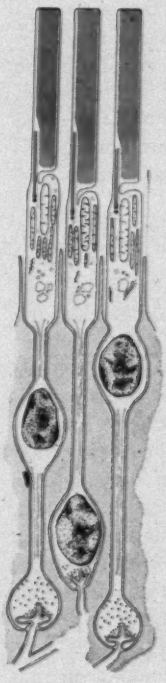
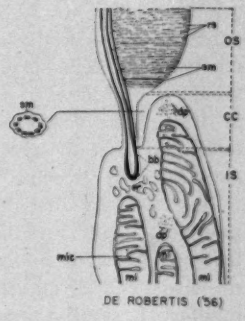


Fig. 7 (Noell). Schematic presentation of three possible cases of phosphate bond energy supply. The upper vertical arrow represents the Embden-Meyerhof pathway, the lower the citric acid cycle. Further explanation in text.

electronmicroscopic finding pertinent to our discussion is the confinement of the mitochondria to the distal half of the inner limb. The proximal region of the inner limb of the rabbit lacks mitochondria completely. There are likewise no mitochondria in the rod fiber, none in the cytoplasm surrounding the nucleus, none at the synaptic end of the rabbit's visual cell (though in rat and mouse a single mitochondrion is there located),[53] and also none in the outer limb. It is a well-established fact that the respiratory system, the array of respiratory enzymes and electron-carriers, is an integral part of the mitochondrial structure. Virtually, the whole respiratory capacity of the rabbit's visual cell, therefore, seems to reside in one small part of the cell. If this part were to support energetically the whole cell, the energy-rich products would have to traverse over consider-





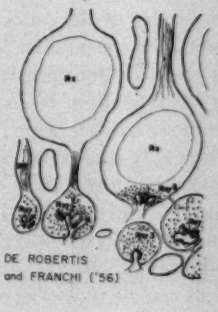


Fig. 8 (Noell). Schematic presentation of the fine structure of the rod cell of the rabbit. (OS-outer segment; cc-connecting cilium; IS-inner segment; mic-mitochondrion; bb-basal body of connecting cilium.) Shaded areas in the drawing on the left represent Müller cell eytoplasm. Processes of the Müller cells extend into the spaces between the inner limbs.

able distances to the sites of utilization. Worst off of all parts, because of its distance from the mitochondria, would be the synaptic region of the rabbit's visual cell where the processes involved in the transmission of excitation to the next neuron probably have special energetic requirements. Therefore, instead of depending upon the supply from a distant center, the parts of the visual cell lacking in mitochondria could be adapted to non-respiratory metabolic support. Indeed, it has been shown that glycolytic enzymes in rabbit and monkey are especially high in the outer plexiform layer.[30, 31, 50]

Thought this compartmentation of the metabolic activities of the visual cell is of very great importance for all considerations of its life processes, it is by no means the only property which distinguishes the life processes of the visual (rod) cell from those of other retinal cells and the body cell in general. The rabbit's visual cell is remarkably sensitive not only to iodoacetate but to oxygen poisoning as well. For example, exposure of a rabbit to a 90 to 100 percent oxygen atmosphere for 30 to 40 hours results in extensive and selective visual cell death, the other retinal cells surviving.[6] Furthermore, though a high dose of x-radiation is needed to destroy the visual cells, this dose is less than half that to which bipolar cells and ganglion cells succumb.[6]

It is unlikely that the effects of oxygen poisoning and x-radiation are mediated through glycolytic inhibition, but rather that the visual cell, as all cells, is dependent on a number of essential processes and this would certainly include processes which require the activity of the mitochondria in the distal part of the inner limb. It is perhaps a consequence of its function as a light receptor that the visual cell is poised in a very delicate metabolic balance which may easily be upset, more easily than in bipolar cells and ganglion cells, and more easily in rod cells than in cone cells in relation to the difference in their functional organization. Nothing at present is known about the dynamics of the synthesis and degradation of essential structural material in the visual cell. I would expect that once knowledge is gained on these points, the unusual vulnerability of the visual (rod) cell will be less mysterious than it appears today.

Visual cell structure in relation to function

The electronmicroscopy of the visual cell, presently a very active field of retinal research, is the foundation upon which present and future studies of visual cell function must be based. I will briefly point out some of the electronmicroscopic findings which are pertinent to the question of possible linkages between metabolism and excitation of the visual cell.

The outer limb where light is absorbed and where the initial events of vision occur is connected to the inner limb by a structure analogous to the ordinary cilium of many plant and animal cells.[27, 51] As shown in Figure 8, the connecting cilium forms a narrow bridge between inner and outer limb and it seems reasonable to assume that the changes which light induces in the outer limb are transmitted through this bridge to the inner limb. The close association of the mitochondria of the visual cell with the connecting cilium suggests a metabolic linkage, involving the mitochondria, between the changes occurring in the outer limb and those transmitted to the other end of the cell. This metabolic link, first proposed by Sjøstrand,[52] may well be involved in the final step of transforming the light induced change into excitation as we usually understand it. The proximal portion of the inner limb, as well as the rod fiber, contains a fibrillar component similar to the neurofibrils of axoplasm, suggesting that these regions are related to ordinary nervous matter.[48]

The synapse between the visual cell and the second retinal neuron is an unusual one. Whereas the synapse between two neurons commonly consists of a close contact of the broadened knoblike ending of the preynaptic fiber with the surface of the postsynpatic element, the visual cell fiber expands at its

end to a spherule into which the dendritic process of the bipolar cell penetrates and branches.[49,53] In a manner typical for synapses, these fingerlike processes are separated from the folding of the visual cell membrane by a narrow gap. The spherule is filled with vesicles, as are the presynaptic sites of most other synapses.[54] In conformity with the present-day thinking on synaptic transmission, these vesicles should contain a transmitter substance which under the influence of excitation is released from the vesicle at the synaptic membrane, diffuses across the gap, and effects a change at the membrane of the bipolar cell element, which then leads to postsynaptic excitation.[55] This process is dependent upon continuous synthesis of the transmitter substance, and the synaptic region of the cell should be expected to contain the machinery for at least the final steps of this synthetic process. Lowry[30,31] has found the outer plexiform layer to be amazingly rich in the initial enzymes of the phosphate pentose pathway, glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase. The inner limb region likewise contains these enzymes in high concentrations but less so than the outer plexiform layer.

Hence, there are at least two sites within the visual cell—at the junction between outer limb and inner limb, and in the synaptic spherule—where biochemical events might be intimately linked to excitation.

As indicated in Figure 8, the visual cells, with the exception of outer and inner limbs, and the other nervous elements of the retina are tightly enveloped by Müller cells.[48–56] All spaces between outer and inner limiting membrane which are not occupied by neuronal structures are filled with Müller cell cytoplasm so that through the entire retinal region between outer and inner limiting membrane no true extracellular space exists, as is case in the brain.[57] The relationship between Müller cell and visual cell, for instance, is so close that rod fiber and synaptic spherule are actually enclosed by two membranes a few hundreds angstroms apart, one membrane provided by the visual cell proper, the other by the Müller cell. A role of the Müller cell in retinal function is certainly indicated, particularly in view of the recent histochemical findings that it is rich in certain dehydrogenases.[58] The Müller cells probably are important elements in the maintenance of retinal homeostasis, comparable perhaps in this function to the pigment epithelium.

Azide response and origin of the c-wave

I would like to discuss now a phenomenon which concerns a functional relationship between the pigment epithelium and the visual cell. Early in my study[3] it was found that the rapid, intravenous injection of sodium azide, in the dark as well as in light, produces a remarkable, almost instantaneous change in the DC potential across the eye (Fig. 9) amounting maximally to 20 millivolts. The time interval between the injection and this DC change was about four seconds and hardly exceeded the time necessary for the blood to carry the azide solution from the vein to the eye. The response could be easily graded by adjusting the concentration of azide and its form depended very much upon the speed of injection. Actually, the rise and fall of the response seemed to represent the concentration of azide in the ocular blood, signaling the passage through the eye of the rapidly injected solution during its first turn through the circulatory system. The reaction to azide was, therefore, not only instantaneous but also rapidly reversible.

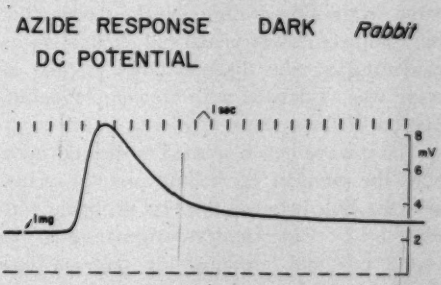


Fig. 9 (Noell). Response of DC potential across the eye to fast intravenous injection of sodium azide, 1.0 mg. in 2.0 ml.

By testing rabbits in which the visual cells had been completely eliminated by a previous administration of iodoacetate, it could be shown that the electrical change in response to azide did not depend upon the visual cells. However, after poisoning with iodate the azide response was lacking. In fact, within 10 minutes after the administration of a high dose of iodate, the response to azide disappeared, although there was no loss or depression of a- and b-waves. The histological effect of iodate is one of preferential or primary damage to the pigment epithelium.[4, 6] For this and other reasons it was deduced that it was the pigment epithelium or a boundary intimately related to it, where azide produced its rise in the DC potential, presumably by interfering with an ion transport system.

Iodate abolished acutely not only the response of the DC potential to azide but it also abolished the c-wave of electroretinogram, that is the slow, cornea-positive potential which follows the b-wave (fig. 10). In fact, all of the effects on the c-wave produced by iodate or other poisons suggested that the c-wave and the response to azide had a common site; moreover, azide markedly and preferentially enhanced the c-wave.

The c-wave by analogy with the azide sensitive components of the DC potential was thus assumed to arise at the pigment epithelium, a view supported recently by the microelectrode studies of Brown and Wiesel.[59] Sudden blockage of the retinal pathway by iodoacetate between the sites of a- and b-wave origin did not diminish the c-wave, but the complete loss of visual cell excitability as evidenced by the disappearance of the a-wave was associated with the complete elimination of the c-wave.

The c-wave hence seemed to depend upon both the pigment epithelium and the visual cell, the link between the two probably represented by ionic changes originating in the visual cell and reaching the pigment epithelium by diffusion.[4]

The analysis went one step further. As

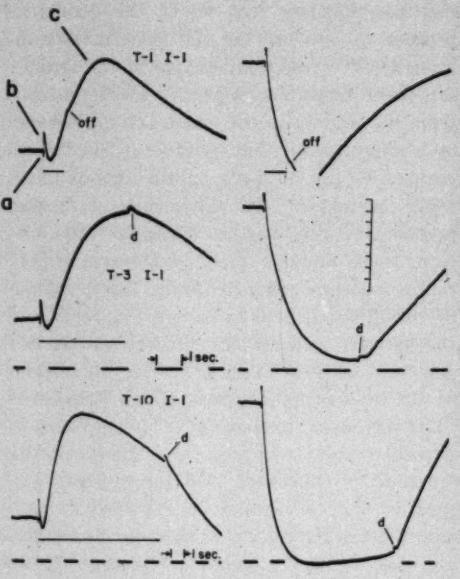


Fig. 10 (Noell). The c-wave of the electroretinogram in response to illumination of various durations (T): 1.0 second for the top record, 3.0 seconds for the middle record and 10 seconds for the bottom record. Note that the tracings of the bottom row are recorded with slower speed. At the left are the normal responses, at the right the responses 20 minutes after iodate administration. (From Noell.[4])

shown in Figure 10 the elimination of the c-wave by iodate "unmasks" a potential of opposite polarity. This slow cornea-negative potential is of very simple form; in response to brief illumination it rapidly reaches its maximal negativity and then slowly and almost exponentially returns to the base line; in response to prolonged illumination, it begins to return toward the base line before the light is turned off. In contrast to the c-wave, this cornea-negative potential was insensitive to azide and therefore termed "azide-insensitive slow potential."

We assume that this cornea-negative potential reflects the original ionic changes resulting from illumination.[4] We further assume that in the absence of iodate, the pigment epithelium attempts by means of active ion transport to restore its ionic equilibrium at the visual cell border, the c-wave being the

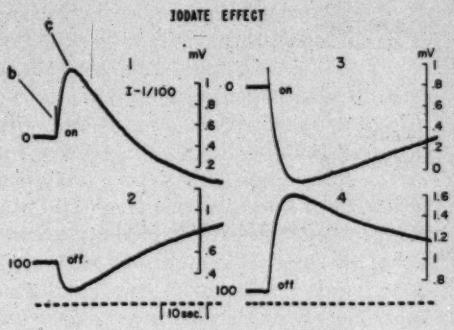


Fig. 11 (Noell). The slow electroretinogram potentials at the "on" and "off" of moderate illumination (no a-wave response). The duration of illumination is 100 seconds; only the first 30 seconds after "on," and the first 30 seconds after "off" are recorded. At left the normal responses, at right the responses about one hour after intraveous iodate. (From Noell.[4])

reflection of this ion transport.

Interestingly, similar events but of opposite polarity occur at the "off" of prolonged illumination.[4] As shown in Figure 11 (left column), sudden cessation of illumination, in this experiment 100 seconds after "on," produces in the rabbit a slow potential change in the opposite direction of the c-wave. If now the animal is poisoned by iodate (right column), and azide sensitivity thus eliminated, the c-wave, as described before, is replaced by the "azide-insensitive" cornea-negative potential while the normal cornea-*negative* "off"-wave is replaced by a cornea-*positive*, "azide-insensitive" reaction. Significantly, the two "azide-insensitive" changes, the "on" and "off" change, have very similar forms and almost the same amplitudes. According to our hypothesis they indicate an ionic imbalance of one sign in reaction to the "on," and of opposite sign to the "off" of illumination. We do not know the nature of the ion species involved or the visual cell reactions responsible, but I expect that further studies of this phenomenon will indicate a close relationship to photochemical events. Recent observations on the light-induced changes in hydrogen ion concentration[60] and in electrical conductance of rhodopsin solutions[61] seem to support this thinking.

There are two general points to this study of the reaction of the pigment epithelium. The first is that a metabolic poison (azide) can instantaneously and selectively interfere with an electrical retinal function. The second is that a non-neuronal cell of the retina can generate an electrical potential as a response to ionic changes occurring in the neighboring neuronal cell in consequence of light stimulation.

Electroretinographic phenomena

The initial part of the electroretinogram comprising a- and b-wave in addition to other components, displays a multitude of normal phenomena depending upon the physical properties of the stimulus and the state of retinal adaptation.[4, 62, 63] Figure 12 illustrates some of the normal variations as recorded in the rabbit. In relation to the multitude of normal phenomena and the complex character of the electroretinogram, the changes produced by drugs and poisons cover a broad spectrum of phenomena, too broad to be reviewed here. Instead I will briefly discuss a- and b-waves, as recorded in response to a

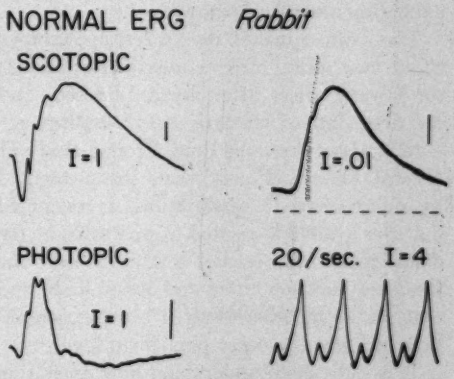


Fig. 12 (Noell). Normal variations of the early fractions of the electroretinogram. I = intensity. The cathode ray is triggered by the light flash (Grass photic stimulator), each dash of the horizontal line represents 10 msec., vertical lines represent 100 microvolts.

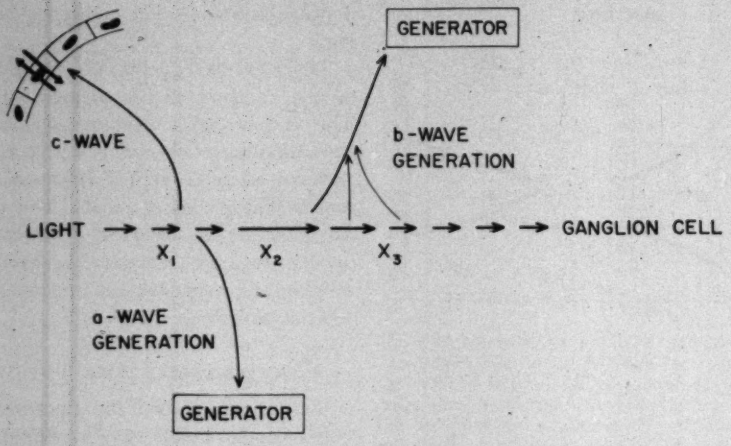


Fig. 13 (Noell). Schematic presentation of retinal pathway and origin of a- and b- and c-wave. "Generator" indicates the unknown structures or boundaries which produce a- and b-waves. The generator for the c-wave is the pigment epithelium.

strong stimulus in the dark, and some of the more instructive changes of these waves resulting from interference with metabolism.

For describing and analyzing the electroretinogram I think of the retinal events as a chain of reactions to which the a- and b-waves are linked at certain steps. As indicated in Figure 13, I prefer to think of these waves as arising from side reactions rather than as representing potentials generating excitation along the pathway.

The a-wave makes its earliest appearance about two msec. after a maximal stimulus; the b-wave arises after about 12 msec., and the first sign of an optic tract response occurs at about the same time. From a study of several abnormal conditions supplemented by microelectrode exploration, I concluded that the a-wave is related to processes at the distal part of the visual cell, at or near the juncture between outer and inner limb, and that the system involved in b-wave generation includes the outer plexiform layer.[4]

It is difficult to understand how excitation of small elements within the retina can give rise to a flow of current which extends throughout the eye. It seems that either a boundary parallel to the retina (such as the pigment epithelium) or a row of closely packed, radially oriented elements, which undergo simultaneously the same change, is required for a phenomenon such as the electroretinogram to arise. It appeared to me at the time of my original studies[4] that the row of inner limbs would be best suited for playing the role of a boundary in the generation of a- and b-waves but I was unable to explain how the inner limbs actually could play this role which, for example, seemed to require their "sensing" of changes occurring in the outer plexiform layer.

Since then all reported attempts to determine the sites of origin of the electroretinogram have exclusively employed microelectrode probings of the retina.[64-70] Opinions formed on the basis of this work differ widely. Tomita[64,65] concluded that the electroretinogram is generated proximal to the visual cells. Brindley[66-68] assumed that a- and b-waves of the frog's electroretinogram appear across a "membrane" in the outer region of the retina, probably the outer limiting membrane, and that the structures producing a- and b-waves "can thus only be the rods and cones."[66] Brown and Wiesel[68] from their recent work on the cat suggest that the

a-wave "comes" from the outer limb region and the b-wave from structures in the region of the outer plexiform layer.

These uncertainties as to the site of origin of a- and b-wave, which cannot be resolved at the present time, should not diminish the usefulness of the electroretinogram to furnish information about normal and abnormal states of retinal function provided one recognizes these uncertainties. Indeed, the lack of such knowledge should rather stimulate the use of drugs and abnormal metabolic conditions in studying the electroretinogram and its relationship to metabolic activity.

Along the chain of intraretinal reactions (fig. 13) poisons may act with preference at one specific area of the scheme or they may act at several points, simultaneously or in sequence. I have indicated three general areas at which the action of a poison may elicit an effect. Blockage at x_1 would depress all activities along the chain. Blockage at x_2 would depress selectively b-wave and optic tract response and since x_2 contains the most time-consuming step or steps in the chain, interference with processes at x_2 would be apt to increase the latency of these responses. Blockage at x_3 would affect the optic tract response without affecting the electroretinogram. The following figures give a few examples.

Figure 14 shows an example of a blockage at a step preceding a-wave generation (area x_1) in which all responses simply vanish while the latency of the optic tract response is not increased. Apparently, the number of responsive visual cells diminishes as if the individual element were affected in an all-or-none fashion. This is the typical effect of iodoacetate in cat, monkey and rat. In the rabbit, a similar rapid decline of all electroretinogram components occurs during high intensity x-radiation of the eye.[5]

Figure 15 shows a typical chronic effect upon the early events of the chain, where the whole electroretinogram is remarkably delayed in its appearance as if processes at x_1 require an abnormally long time to elicit the first sign of an electric reaction. This change occurs in association with the degeneration of outer and inner limbs. It also is a typical early phenomenon of abnormal retinal function in hereditary visual cell degeneration of mice.[6]

Figure 16 gives another example of the iodoacetate effect in the rabbit using a high dose. The chain appears to be blocked first at x_2, b-wave and optic tract response diminish

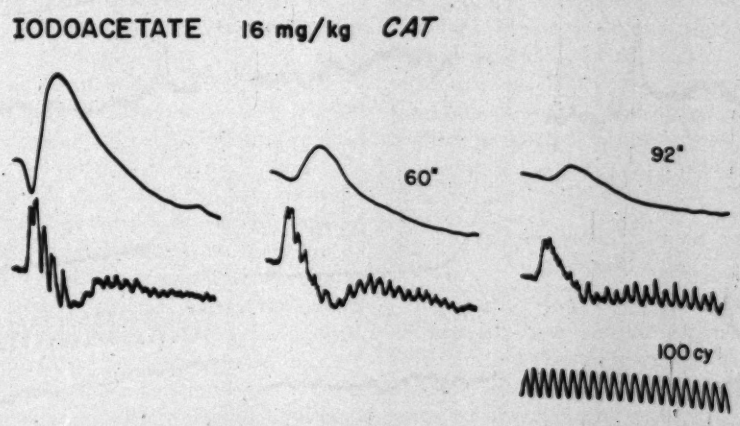


Fig. 14 (Noell). Effect of intravenous iodoacetate on electroretinogram and optic tract in cat. (From Noell.[1])

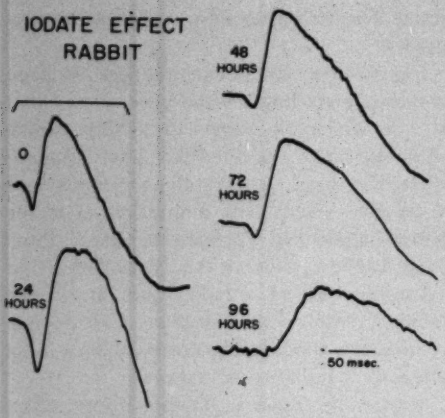


Fig. 15 (Noell). Effect of iodate on a- and b-wave measured 24 to 96 hours after intravenous administration. (From Noell.[4])

while their latency increases. The rapid decline of the b-wave is accompanied by the emergence of the a-wave in the form of an extraordinarily high and long lasting electrical change, supposedly because b-wave elimination "unmasks" the a-wave. As a second consequence of the action of the poison, x_1 is affected and the a-wave disappears simultaneously with a notable increase in latency.

The next example, Figure 17, is more complicated; it shows the effect of a moderate dose of iodoacetate in the rabbit. There is first a decline of the "off" wave of the optic tract response, associated only with a slight electroretinographic change as if at or beyond x_3 mechanisms essential for the generation of the "off" effect (inhibitory mechanisms) were disturbed.[4, 71]

Somewhat later the typical increase in the latency of b-wave and optic tract response occurs, associated with a lowered peak amplitude of the b-wave if measured from the baseline. While it seems that this effect at x_2 has become stabilized about three minutes after the injection of the poison, the whole

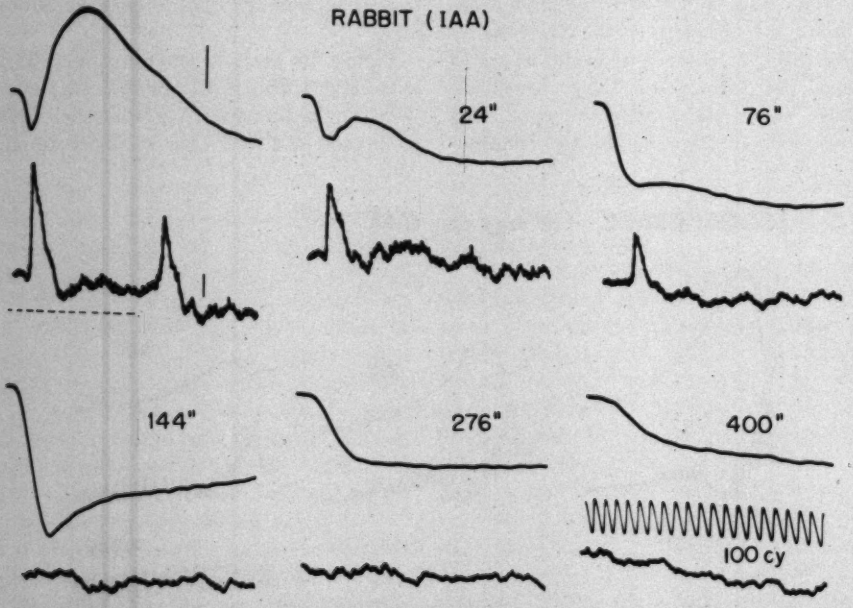


Fig. 16 (Noell). Acute effect of intravenous iodoacetate on electroretinogram and optic tract potentials in the rabbit. Note the on and the off responses of the optic tract in the control tracing. The effect of iodoacetate is already pronounced 24 seconds after the injection. (From Noell.[1])

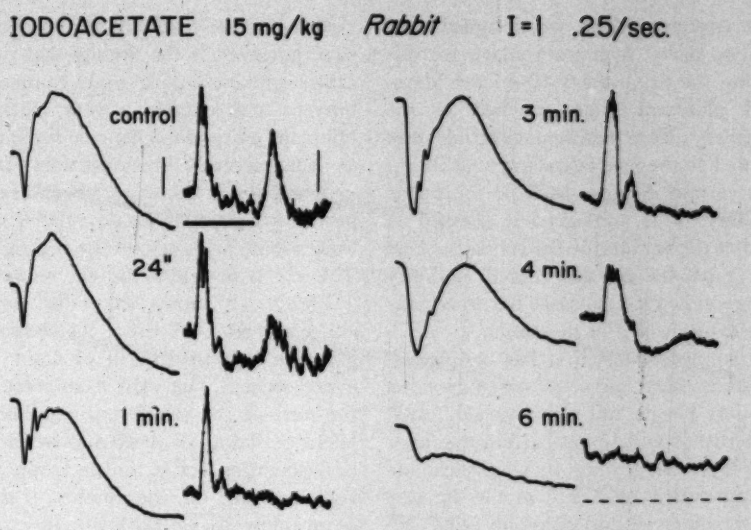


Fig. 17 (Noell). Effect of iodoacetate on ERG and optic tract response. The changes develop slower than in Figure 16.

chain suddenly begins to fail as if a system common to all now completely breaks down.

It is not possible yet to relate these effects and those of several other poisons to the structures and metabolic systems involved. They indicate a high susceptibility of the chain at certain points, and in a general way they seem to support the concept outlined earlier that metabolic reactions might be an intimate of visual cell excitation at certain sites.

It should be mentioned that the steps of the chain located between a- and b-wave origin are those most easily and most rapidly affected by a variety of chemical agents including for example azide and barbiturates.[6]

Species differences involve mainly the effect of anoxia and iodoacetate upon the b-wave and they are between the rabbit on the one hand and cat, rat, monkey on the other; these differences hence seem to coincide with differences in the vascularization of the inner layers and in the relationships between glycolytic and respiratory activities therein; they perhaps may one day be found to relate to the presence or absence of a mitochondrion in the synaptic spherule. At any rate they would suggest, as do other experimental results, that a retinal region at or even vitread the proximal end of the visual cell participates in b-wave generation.

Obviously, the effects upon a- and b-waves will provide only a vague outline of relationships between function and metabolism, simply because the affected system is too large. We have recently begun efforts to circumvent this difficulty and are testing a technique which perhaps permits observations on a finer scale. In closing, I would like to describe now briefly our present use of this technique at the level of the retinal ganglion cells.

Metabolite effects upon retinal ganglion cells

In order to study drug effect upon the inner synaptic layer and the ganglion cell, we have adapted the technique of the electrophoretic injection of drugs with microcapillaries which in recent years has been successfully employed in the analysis of neuromuscular and spinal transmission.[72-74] The

electrode assembly which we designed consists of two parts: a microcapillary for administering the drug and a 10 micron glass-insulated platinum wire attached to the microcapillary. The platinum electrode was so cemented to the microcapillary that its tip was at a certain distance back of the tip of the capillary. This arrangement allowed us to record at the surface of the retina the best defined responses of the retinal pathway (the ganglion cell discharges) but to administer the drug deeper in the retina.

We attempted to test first the significance of the histochemical findings,[75] based upon the technique of Koelle and Friedenwald,[76] that cholinesterase is widely localized in the inner synaptic layer suggesting that acetylcholine is the transmitter substance at the synapse between bipolar cell and ganglion cell.[77] Indeed we obtained evidence in support of this theory.[78]

More to the point of our present discussion, however, is the finding that two amino acids, glutamic acid and gamma aminobutyric acid, exerted a very marked effect upon the ganglion cells in a similar manner as was recently demonstrated for spinal neurons by Curtis, et al.[79, 80] Indeed, the reports by Curtis, et al. stimulated us to test these amino acids upon retinal ganglion cells. The effects proved to be very remarkable.

Figure 18 shows the effect of gamma aminobutyric acid upon the response of a ganglion cell to a flash of light delivered every second. The light flash coincides with the start of the sweep and provokes after a latent period of about 40 milliseconds a burst of discharges. After the electrophoretic current is turned on, the number of discharges in response to the flash decrease second by second and become abolished. After turning the current off, the discharges slowly recover

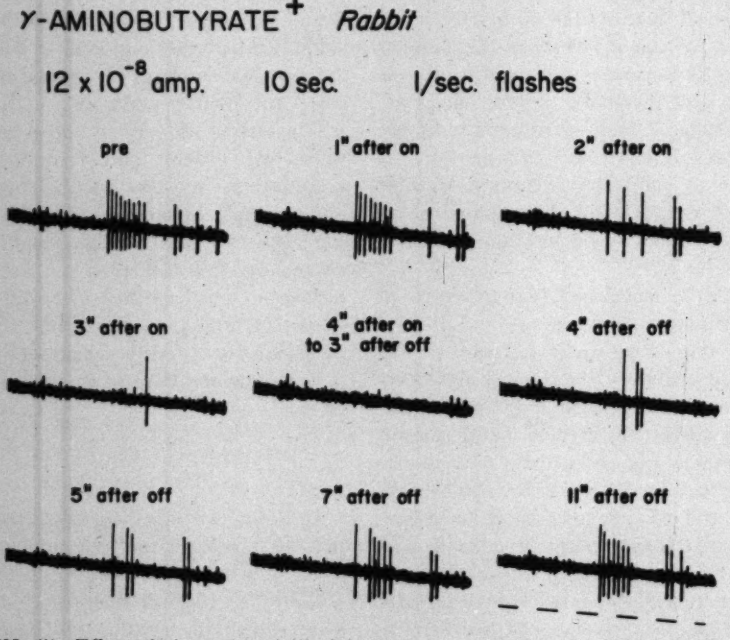


Fig. 18 (Noell). Effect of electrophoretically injected gamma aminobutyric acid on the discharges of a retinal ganglion cell in response to brief illumination at the start of the sweep. "On" and "off" refer to the start and end respectively of the application of the electrophoretic current. (From experiments by Noell and Lasansky.)

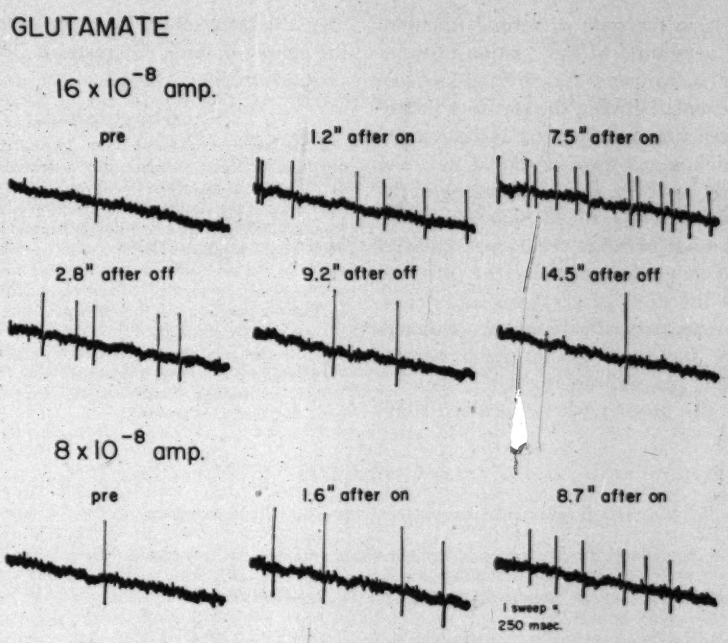


Fig. 19 (Noell). Effect of electrophoretically injected glutamate on spontaneous activity of a retinal ganglion cell. Bottom row illustrates effect at one half the intensity of the electrophoretic current as used in recordings at top. (From experiments by Noell and Lasansky.)

over a period of 10 seconds. The effect can be provoked in the same manner whether the amino acid is administered as an anion or a cation.

Figure 19 illustrates the effect of glutamate which is the opposite of that of gamma aminobutyric acid. Prior to the administration of glutamate, the ganglion cell under observation is spontaneously active at a rate of less than one spike per cathode ray sweep. Almost immediately after the electrophoretic current is turned on, its activity is increased and continues to increase for several seconds. When current is turned off the spontaneous activity returns to normal within about 10 seconds.

Curtis, et al.[80] interpret these effects as resulting from the direct interaction of these amino acids with a component of the nerve cell membrane. Our findings are in support of this view. But their main significance it seems to me is the demonstration that common metabolites can in a specific way influence nerve cell activity.

Concluding remarks

Where do we stand then concerning the life processes of the visual cell? We are at a stage where very much is known about the fine structure of the cell. We even know the distribution of several key enzymes over the visual cell. Our knowledge of the photochemical processes has been developed to a very high degree, mostly due to the elegant studies of Dr. Wald and his group. I think we have also a better picture of the metabolic capacities of the retina. We apparently can relate structural organization and metabolism with certain aspects of cell maintenance, as illustrated by the effect of a metabolic poison. Where we are lacking is in the understanding of the electrical phenomena of the retina, which are such an important tool for detect-

ing changes in the state of retinal function. We know very little of the relationships between metabolism and function and of the series of events between the primary photochemical reaction and nervous excitation, up to the ganglion cell level. I should not miss mentioning, however, that a beginning in the latter areas has been made by Svaetichin.[81] To enlarge the list of what we do not know, I would like to stress the absence of information about the rates of synthesis and degradation of essential cell components, although techniques for such studies have become available and are utilized for other tissues. I hope that the phenomena I discussed today may stimulate others to include the retina in the sphere of their interests.

666 Elm Street (3).

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GLIA OF THE HUMAN RETINA*

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It seems to have been the fate of the intercellular supporting structures to have been more or less ignored by the early histologists. This may explain why our knowledge of the glia of the retina is so limited. The neurons of the retina found much attention by early authors and details of their intricate structure and arrangement are already know for more than a hundred years. The retinal glia, however, is still virtually unknown and not very long ago one author wrote in a paper that there is no other glia than the radial fibers of Mueller in the retina. You will be able to decide for yourself after I am through with this talk whether he was right or not.

* From the Department of Ophthalmic Surgery and the Laboratory of Neuropathology of the University of Michigan Medical Center. Supported by grants 2B-5163(C) and B-475-C5 of the United States Department of Public Health, Education and Welfare.

The present paper represents a kind of a summary of the results of our studies of the glia of the human retina that were done continuously through the last six years. It is a continuation of and an addition to the earlier talks on the glia of the human eye that were given before this same meeting in 1955,[1] 56,[2] and 57.[3]

The supporting structures of the human retina are classified as neuroglia. Three main types of neuroglia are found in the retina: the radial fibers of Mueller, the astroglia and the perivascular glia. It must be emphasized that the neuroglia is *not* mesodermal in origin and has nothing in common with the mesodermal connective tissues. Neuroglia has been shown to develop from the primitive medullary epithelium of the optic vesicle which in the early phases of embryonal development differentiates into neuroblast and glioblasts. The glioblasts are the percursers of the retinal neuroglia.

It is somewhat confusing that other elements of the human retina which are very different in their structure and functions are also known as glia. These are the microglia. It must be emphasized that microglia undoubtedly are mesodermal in origin. Microglia are found in all parts of the central nervous system. They were first described by del Rio Hortega[4] and are therefore also called Hortega cells. They are wandering phagocytes and can be considered as the histiocytes of the nervous system.

A demonstration of the different types of glia found in the human retina and some of their common reactions and pathology is given in the following:

Material and method

The material used for this demonstration is that of the human eyes that were examined in this Eye Pathology Laboratory. Frozen sections of parts of the retina of each eye were stained with the silver carbonate methods of del Rio Hortega.[5] These are the only stains that have proven to allow for a consistent and complete demonstration of the retinal glia. The silver stains were used only as additional stain on small parts of the retina of all eyes while most of each globe was imbedded in paraffine and studied with the accepted methods of routine eye pathology.

All illustrations presented in this paper are unretouched photomicrographs.

Histologic demonstration and comments

1. Astroglia

The single cells of the astroglia are called astrocytes since their cell bodies are star-shaped. Figure 1 shows an astrocyte of the normal human retina as seen in a silver-stained flat section. The astrocytes have a rather small round nucleus and long cellular processes. They form a networklike system in the inner layers of the retina. Some of the processes of the astrocytes surround the ganglion cells and have close contact with their neurites and dendrites and their cell bodies. Other processes form sucker footlike formations on the wall of small blood vessels (fig. 1-B, c). The astroglia are arranged as a dividing system between the retinal neurons (neuroectodermal in origin) and the blood vessels and their connective tissues (mesodermal in origin). There is no direct contact between the neurons and the blood vessels of the normal retina. It seems to be the function of the astroglia not only to support the neurons but also to select the nutritional fluids for the neurons from the blood vessels and to transport them within their protoplasm to the neurons. It seems that this function is the main purpose of the retinal astroglia since astroglia is only found in those retinal layers where there are blood vessels: the nerve fiber layer, the ganglion cell layer, and the inner plexiforme layer. The inner nuclear layer has no astrocytes of its own. However, processes of the astrocytes of the inner plexiform layer always surround and accompany the capillaries as they enter the inner nuclear layer.

It is important to realize that the retinal astroglia is not very different from the astroglia of the central nervous system. This

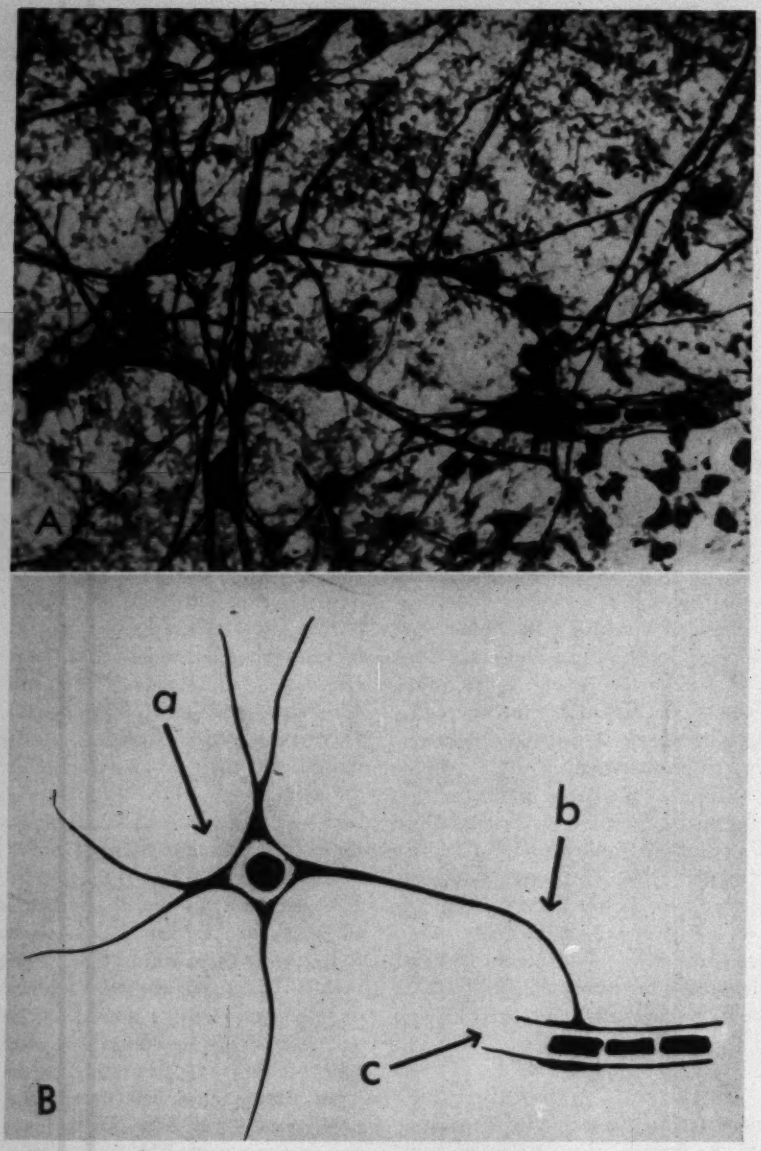


Fig. 1 (Wolter). (A) Flat section through inner plexiform layer of normal human retina. A typical astrocyte is seen with its long processes one of which ends on the wall of a capillary filled with erythrocytes. (B) A drawing of the same astrocyte: (a) Star-shaped cell body with processes. (b) Sucker-foot-like process on capillary. (c) Blood capillary. (A. Frozen section, Hortega stain, photomicrograph.)

is not surprising since the retina actually is a part of the central nervous system. Figure 2 shows astrocytes (a) of the normal human brain. These cells also show the typical long intertwined processes (b) and their sucker footlike endings on the wall of blood vessels (c). The astrocytes of the brain are closely related to those of the retina. However, the

Fig. 2 (Wolter). Astroglia (arrows) with sucker-footlike processes of blood vessels in white matter of normal human brain. (Frozen section, Hortega stain, photomicrograph.)

latter are somewhat modified and adjusted to the special layer like arrangement of the neurons and the blood vessels in the retina.

In the normal adult the astroglia form a very regular honeycombed system all through the inner layers of the retina. This network is somewhat denser and the single astrocytes are somewhat larger in babies. In senescence the astroglia show a decrease of the number of glial processes (fibers) and the remaining fibers are coarser. Figure 3 shows the glial network in the retinal of a normal eye of a 106-year-old man. The astrocytes in this case are small and they have lost some of their processes. The remaining processes are coarse and irregular.

A multitude of pathologic changes of the retinal astroglia can be observed in eye diseases. All of the changes that we have seen so far are nonspecific. Details of the different types of reaction and degeneration of the retinal astroglia were reported in earlier papers.[1,6-9] Hyalinization with the formation of different permanent hyaline structures in the retina is the most common type of degeneration seen in chronic eye diseases. In acute diseases it is more common to see liquifaction and disappearance of the retinal astroglia. The reactions of the astroglia can usually be understood if their close relations to the retinal neurons are considered. In the early and less severe phases of pathologic processes the astroglia tries to protect the neurons and may be found swollen and hypertrophic. In the late and severe stages the astroglia forms a scar and fills the space of the destroyed neurons. Or it may be destroyed together with the neurons and cystic degeneration then results. Different phases of proliferation and scar formation of retinal astroglia are demonstrated in Figures 4 to 7.

Figure 4 shows the network of astroglia in a flat section of the retina of an eye with advanced hypertensive retinopathy. Moderate hypertrophy and proliferation is seen. But the normal networklike arrangement is well preserved.

Figure 5 shows the astroglia of the inner retina of an eye with absolute hemorrhagic glaucoma following venous occlusion. The astroglia in this picture are even more hypertrophic and the astrocytes have many long

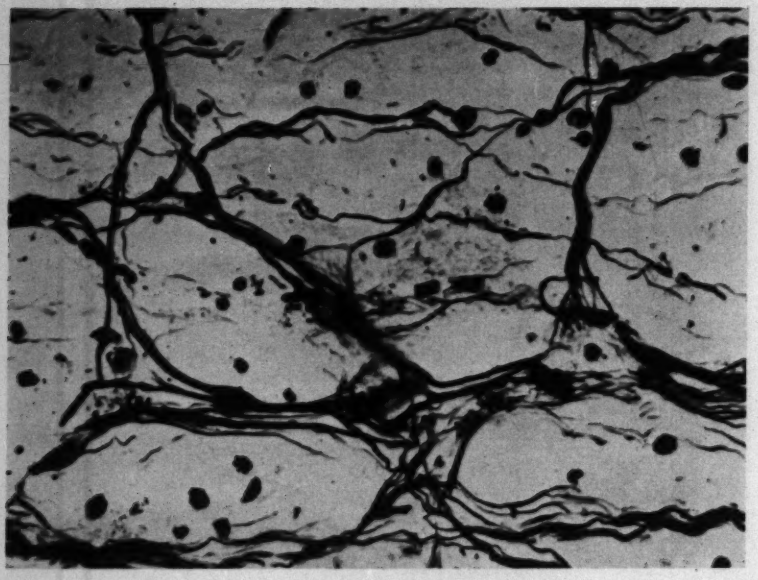


Fig. 3 (Wolter). Flat section through inner plexiforme layer of the retina of the normal eye of a 106-year-old man. The glial processes are coarse. (Frozen section, Hortega stain, photomicrograph.)

Fig. 4 (Wolter). Flat section through inner plexiforme layer of the retina of an eye with advanced hypertensive retinopathy. The astroglia show moderate hypertrophy and proliferation. (Frozen section, Hortega stain, photomicrograph.)

Fig. 5 (Wolter). Flat section through ganglion cell layer of the retina of an eye with old secondary glaucoma following venous occlusion. The astroglia form a dense scarlike network. (Frozen section, Hortega stain, photomicrograph.)

and powerful processes. The normal honeycombed architecture of the retina, however, is still preserved.

Figure 6 shows a very advanced stage of retinal gliosis in the detached and shrunken retina of a 12-year-old boy who had a perforating injury. In this case all of the inner retina is composed of densely arranged glial fibers (a). Cross sections of the inner parts of Mueller's radial fibers are seen in the spaces between the proliferated astroglia (b).

Figure 7 shows finally an area of proliferated retinal astroglia in the detached and shrunken retina of an old case of phthisis bulbi. The architecture of the normal retina is completely lost in this case. All that has remained of the retina is a dense and irregular scar of astroglia.

It was seen in Figure 1 that the astrocytes normally have close relations to the blood vessels. They virtually surround all retinal blood vessels with their sucker footlike processes. Hyperplasia of the astroglia results in increased density of the astrocytes around all blood vessels. A dense system of astrocytes also develops around new formed blood vessels as they may be found for example following venous occlusions in the retina (fig. 8). Figure 9 shows that numerous astrocytes are also found to surround retinal microaneurysms. These findings are not surprising since we already emphasized that the astroglia represent a barrier between the neurons and the blood vessels of the retina.

The astroglia of the nerve fiber layer are distinctly different from those of the other layers of the inner layers. They are bipolar cells with elongated nuclei and two long straight processes with accompany the neurites of the nerve fiber layer (fig. 10). These cells are identical with the so-called elements of Remak or lemmocytes.[10] There is no doubt that they represent the astroglia of the nerve fiber layer which is adjusted to the special architecture of this layer. Not all lemmocytes are of the simple bipolar structure of the one seen in Figure 10. Some of these cells may show branching processes and there are transitional forms at the limit between nerve fiber and ganglion cell layers which look somewhat like astrocytes. When lemmocytes proliferate under pathologic con-

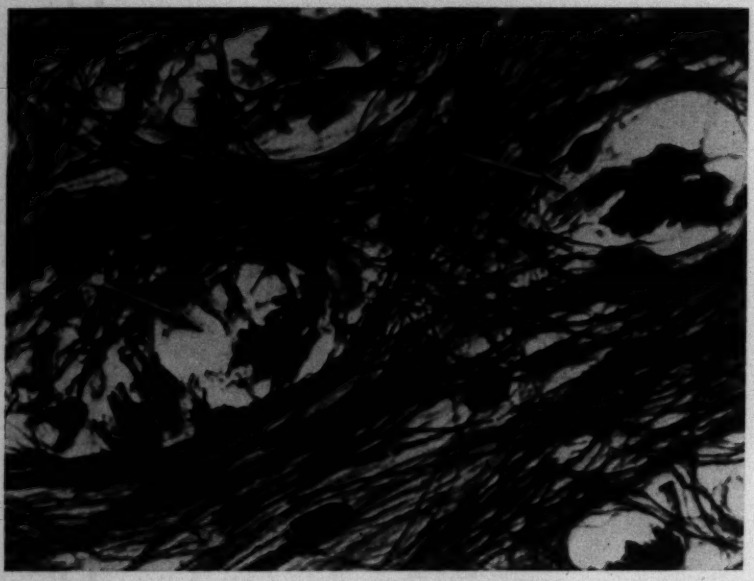


Fig. 6 (Wolter). Flat section through inner layers of retina of the eye of a 12-year-old boy with old perforating injury. The astroglia have replaced all other structures of the inner retina except for Mueller's radial fibers which are seen in cross section (arrows). (Flat section, Hortega stain, photomicrograph.)

Fig. 7 (Wolter). So-called proliferating astroglia of inner retina in the detached and shrunken retina of an eye with old retinal detachment. (Frozen section, Hortega method, photomicrograph.)

Fig. 8 (Wolter). The dense system of astroglia around a new-formed thin-walled vein in the retina of a case of occlusion of the central vein. (Frozen section, Hortega stain, photomicrograph.)

Fig. 9 (Wolter). A dense system of astroglia around a retinal microaneurysm as seen in a case of old occlusion of the central vein. The capillary aneurysm shows proliferation of its endothelial cells in the center and hyalinization of its walls. (Frozen section, Hortega stain, photomicrograph.)

Fig. 10 (Wolter). Flat section through nerve fiber layer of normal human retina with a typical lemmocyte (arrow). These cells represent small elements with two long glial processes in a bipolar arrangement. (Frozen section, Hortega stain, photomicrograph.)

ditions they usually take a star-shaped form and cannot be differentiated from the astrocytes of the deeper layers. It is relatively rare to see proliferation of lemmocytes in their original shape and arrangement as seen in Figure 11 of an area of the inner retina of an eye with advanced chronic simple glaucoma.

The inner limiting membrane is the innermost layer of the retina. This membrane is composed of two parts: (1) the inner brushlike ends of the radial fibers of Mueller (fig. 12) and (2) a homogeneous substance that is well visible in Figure 13. The ends of the radial fibers form a mosaiclike pattern (fig. 12) which is reinforced and interconnected by this homogenous substance. The structure of the inner limiting membrane can be especially well demonstrated in cases with retinal folding as often seen in retinal or retroretinal tumors or in shrinking retinal scars (fig. 14). The inner limiting membrane is considered part of the system of Mueller's fibers. It is therefore not surprising to find that the only area of the retina that has no radial fibers of Mueller also has no inner limiting membrane. This area is the optic disc. Figure 15 clearly shows that the inner limiting membrane ends at the margin of a normal optic disc. In the place of the fibers of Mueller the optic disc has a special type of astroglia with long spiderlike processes: the so-called "spider cells."[11] These cells are astrocytes that are adjusted to serve for support, nutrition and protection of the nerve fibers of the optic disc. Figure 16 shows the surface of a normal optic disc at high power. There is no inner limiting membrane and the dense layer of processes of the spider cells represents the inner surface of the optic disc.

The fact that there is no inner limiting membrane to cover the glial structures of the optic disc is of some importance. This allows for some peculiar glial cells to grow out of the optic disc onto the inner surface of the retina and there to multiply under certain conditions. These cells on the inner retinal surface have been known for a long time and have, for example, by Friedenwald[12]

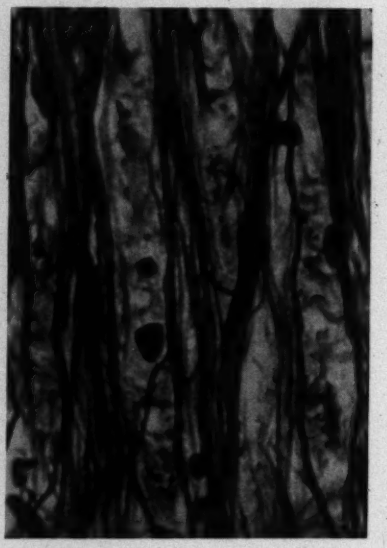


Fig. 11 (Wolter). Flat section through nerve fiber layer of the retina of an eye with old chronic simple glaucoma. Proliferation of lemmocytes is seen. (Frozen section, Hortega stain, photomicrograph.)

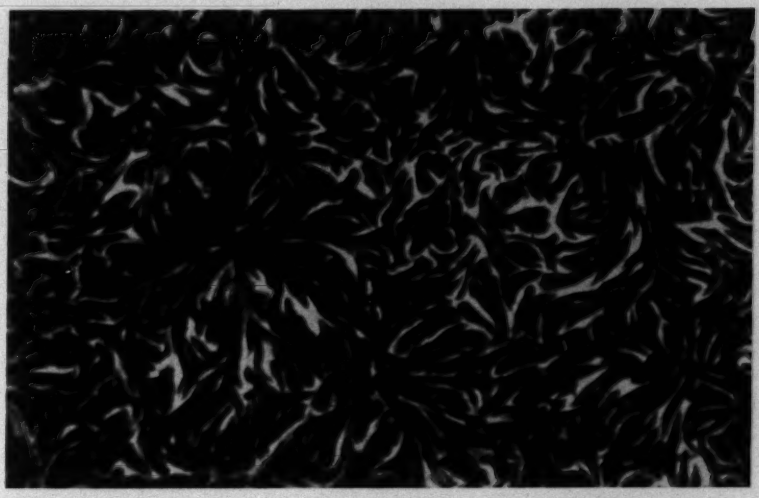
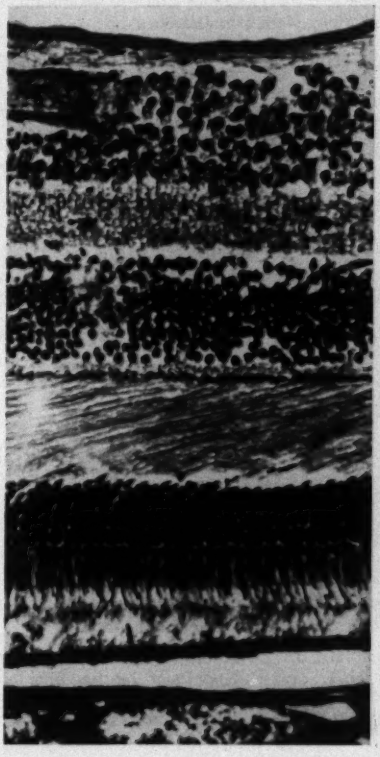


Fig. 12 (Wolter). Flat section through the inner limiting membrane of the retina of a two-month-old child. The glia stain shows the mosaic of the inner ends of Mueller's radial fibers in this layer. (Frozen section, Hortega stain, photomicrograph.)



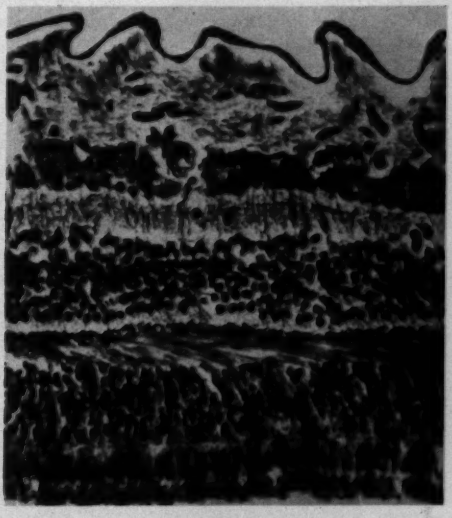


Fig. 14 (Wolter). Folding of inner limiting membrane next to an old retinal scar. (Cross frozen section of retina, Hortega stain, photomicrograph.)

Fig. 13 (Wolter). Cross section through the retina of a normal human eye. The inner limiting membrane is well visible as a black-stained layer. (Frozen section, Hortega stain, photomicrograph.)

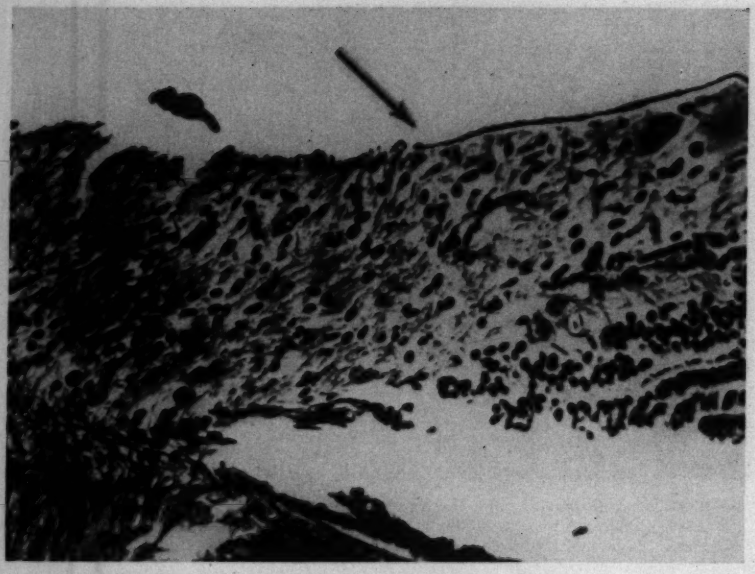


Fig. 15 (Wolter). Cross section through the retina at the optic disc. The inner limiting membrane ends at the disc margin (arrow). (Frozen section, Hortega stain, photomicrograph.)

Fig. 16 (Wolter). High-power view of the surface of the optic disc of a normal eye in a cross section. This is formed by a dense layer of processes of spider cells. There is no inner limiting membrane. (Frozen section, Hortega stain, photomicrograph.)

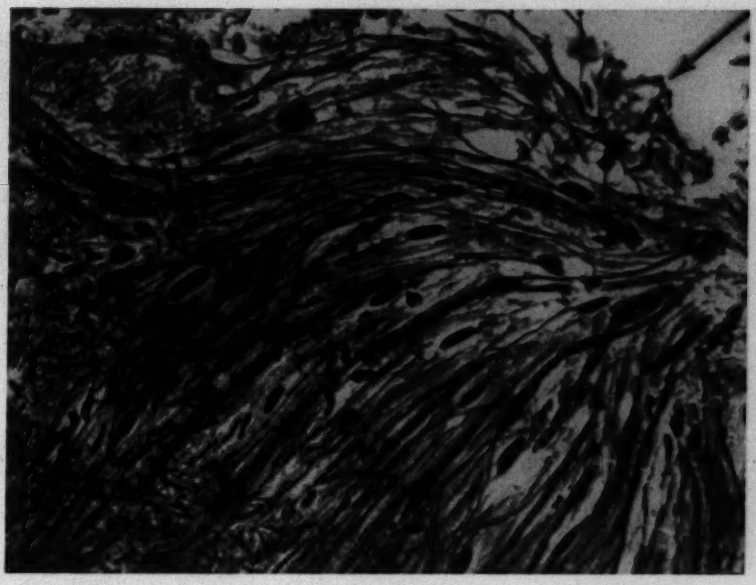


Fig. 17 (Wolter). Dense layer of new-formed glial cells on the inner limiting membrane of the retina at the disk margin (arrow) in a case of old secondary glaucoma. It appears as if these cells have grown out of the optic disc area. (Frozen section, Hortega stain, photomicrograph.)

(and personal communication) been called "endothelium like cells." Our stains show that they actually are astroglia. Some of these cells are found normally on the inner limiting membrane of normal eyes in senescence.[13] They are usually limited to the posterior pole. In degenerative and old inflammatory diseases, however, these cells start to proliferate and often form a whole new layer of glia on the inside of the retina. Figure 17 shows a dense layer of such cells radiating from the margin of the optic disc of an eye with old secondary glaucoma. Figure 18 shows giant glial cells within such a new layer on the inner retinal surface in a case of old open-angle glaucoma. The thick membrane of these cells seen in Figure 19 was found in a case of absolute hemorrhagic glaucoma following venous occlusion.

It may be mentioned that the proliferation of astroglia on the inner surface of the retina can often be recognized with the ophthalmoscope at clinical examination. The thin membrane appears as a whitish superficial reflex which is called "glial chagrin" by some examiners.

2. Radial fibers of Mueller

I have found the radial fibers of Mueller usually to be relatively passive as compared to the more reactive astroglia. Figure 20 shows the surviving framework of the Mueller fibers in the retina of a case of old retinal detachment. All the neurons and the astroglia are destroyed in this retinal area. Some necrotic preservation of nuclei of the inner and outer nuclear layer is seen. However, the fibers of Mueller still show virtually no reaction except for some atrophy. Figure 21 shows a folded area of the retina in a case of nonspecific endophthalmitis. The severe retinal damage in this case has resulted in some destruction of Mueller's elements in the center of the picture. A type of cystic retinal degeneration has resulted.

Figure 22 represents a section through the retina of an advanced case of phthisis bulbi. Parts of the radial fibers of Mueller are seen

Fig. 18 (Wolter). New-formed layer of glia on the inner retina in a case of old open angle glaucoma. One multinucleated glial cell is seen (arrow). (Frozen flat section, Hortega stain, photomicrograph.)

in the lower half of the picture. These fibers are coarse and definitely compressed. But they still have about their normal arrangement and show no proliferation. The astroglia in the upper half of the picture shows extensive hypertrophy and proliferation. It can be said that the radial fibers of the retina are the most resistant retinal elements which disappear last in degenerative processes. However, they are mainly supporting

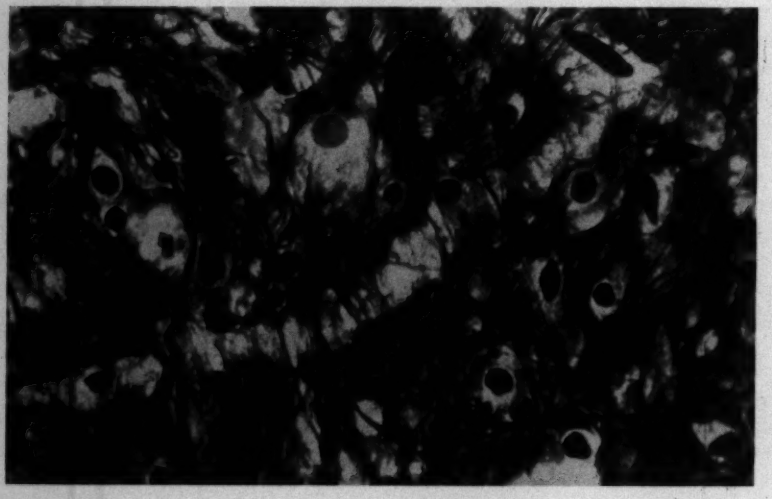


Fig. 19 (Wolter). Very dense layer of new formed glia on the inner retinal surface in a case of old hemorrhagic glaucoma. (Frozen flat section, Hortega stain, photomicrograph.)

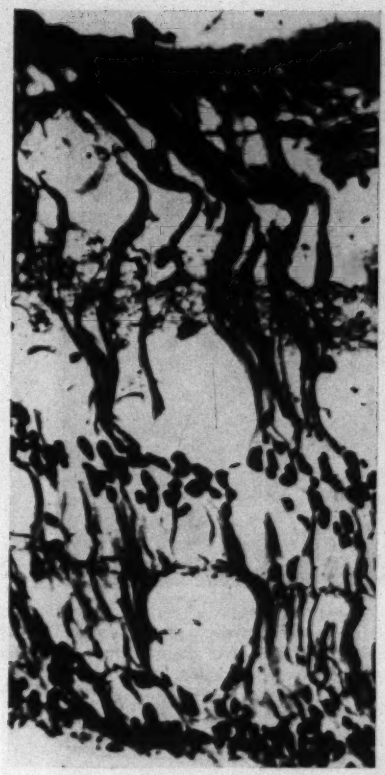


Fig. 20 (Wolter). Cross section of retina in a case of old retinal detachment. The astroglia and most of the neurons are missing. Only the framework of Mueller's radial fibers has survived. (Frozen section, Hortega stain, photomicrograph.)

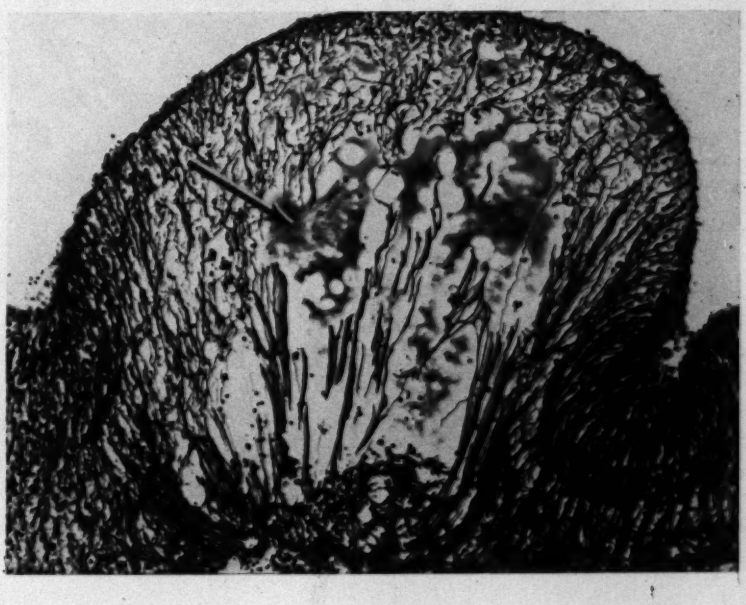
in function and rarely show active pathology.

A peculiar type of columnar cells is seen in many cases of advanced scarification of the retina. These cells stain with the techniques for the demonstration of the glia and they often form bands and rosettes.[14] We do not know yet what these cells are. There is a possibility that they represent active radial fibers of Mueller. But this is not at all certain.

Figure 23 shows a well developed rosette formation of cells of this type which was found in a case of advanced retinitis pigmentosa.

Below:

Fig. 21 (Wolter). Cross section of the folded retina of an eye with endophthalmitis. Partial destruction of the neurons, the astroglia and Mueller's radial fibers is seen in a central area (arrow). (Frozen section, Hortega stain, photomicrograph.)



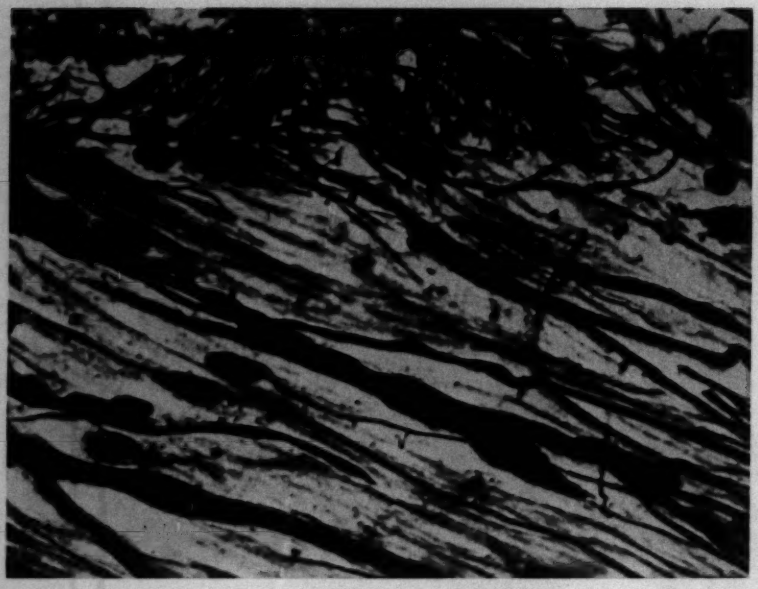


Fig. 22 (Wolter). Cross section through the detached and shrunken retina of a case of phthisis bulbi. Hypertrophic astroglia are seen in the upper part of the picture. Compressed parts of the radial fibers of Mueller are seen in the lower half. Mueller's fibers are coarse but still have their normal arrangement and show no proliferation. (Frozen section, Hortega method, photomicrograph.)

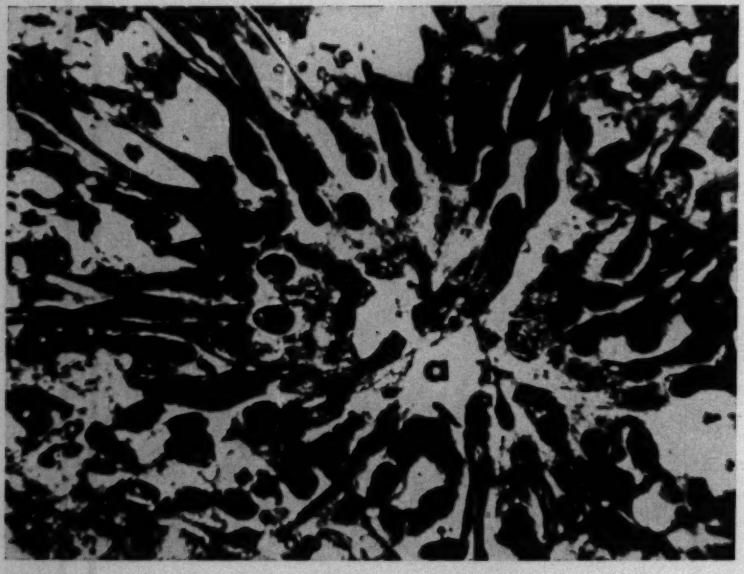


Fig. 23 (Wolter). A rosette formation in the scarified retina of an advanced case of retinitis pigmentosa. (Frozen section, Hortega stain, photomicrograph.)

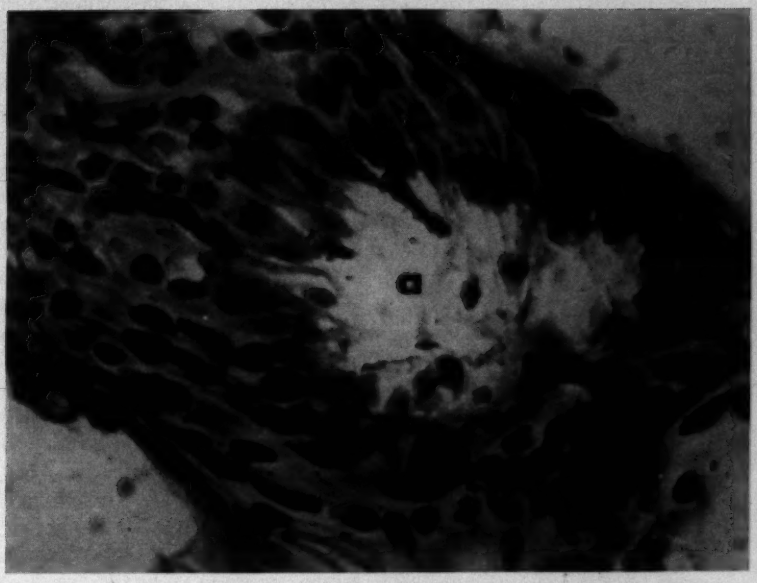


Fig. 24 (Wolter). Flat section of the peripheral retina with cystoid degeneration and a rosette formation (a). (Frozen section, Hortega stain, photomicrograph.)

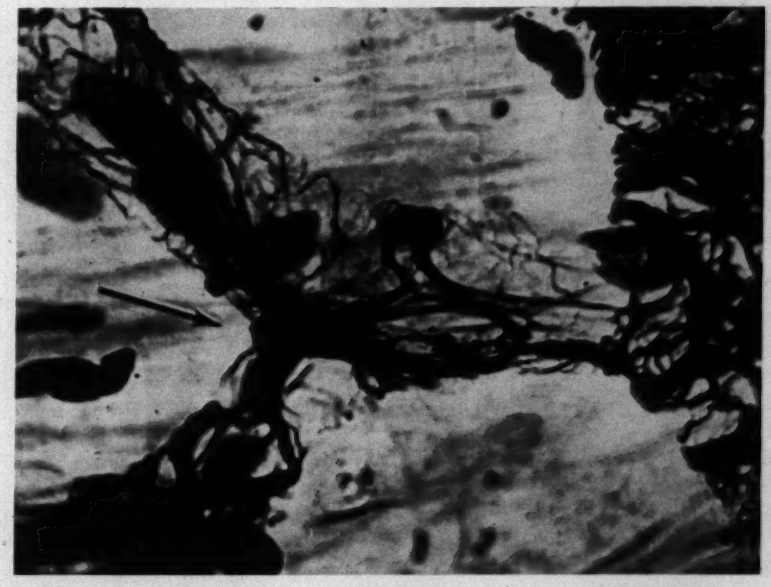


Fig. 25 (Wolter). A normal perivascular glia cell around a capillary of the human retina (arrow). Its long processes surround the small blood vessel. (Frozen section, Hortega stain, photomicrograph.)

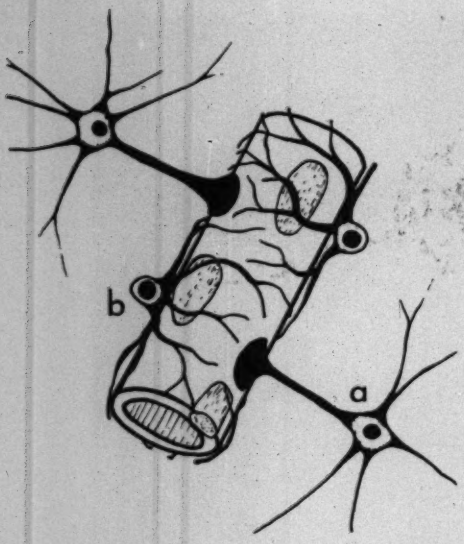


Fig. 26 (Wolter). A drawing to demonstrate the arrangement of the retinal astroglia (a) and the perivascular glia (b) in relation to the retinal capillaries.

Figure 24 shows a somewhat similar rosette seen in the periphery of a normal retina within an area of cystoid degeneration. We believe that this latter rosette is equivalent to the rosettes of the peripheral retina described by Teng and Katzin.[15, 16] They are composed of very similar columnar cells than the rosettes found in retinal scars and primary vitreous attachments are found in the center of these peripheral rosettes.

3. Perivascular glia

This peculiar type of glia is found only around the capillaries of the human retina. Because of staining characteristics, shape and arrangement it is our definite impression that the perivascular glia is a special type of neuroglia.[17] This means that we consider it to be of neuroectodermal origin. Liss[18, 19] has found the same cells in the olfactory bulb and in the neurohypophysis and also considers them neuroectodermal in character. The perivascular glia of the normal human retina represent star-shaped cells with many long processes which surround the wall of the capillaries (fig. 25). Under normal conditions all processes of these cells are found attached to the blood vessel wall. The cell bodies are much like those of the retinal astroycytes and have a similarly small round nucleus.

Figure 26 represents a drawing reconstruction which shows the arrangement of the normal perivascular glia and their relations to the other elements of the retinal capillary wall. It was pointed out above that there is nowhere in the normal retina direct contact of the neurons with the mesodermal tissues of the retinal blood vessels. The limitation of these two tissues of different origin is achieved in part by the sucker footlike processes of astrocytes which cover most of the wall of the capillaries. It is our impression that those areas of the capillary wall that are not covered by the sucker footlike processes of the astrocytes are surrounded by the processes of the perivascular glia. The perivascular glia are a normal element of the human retina. Their function, however, is not yet understood.

Proliferation of the perivascular glia is a very common pathological finding in all kinds of chronic and degenerative eye diseases. The cellular processes become very long and coarse and form bizarre structures around the blood vessels (fig 27). These processes then also extend into the retinal tissues and are no longer confined to the blood vessel wall. It is common to find survival of hypertrophic perivascular glia which often remain in the retina after the blood vessels to which the glia originally belonged has already disappeared by degeneration (fig. 28). Proliferation of perivascular glia is found not only in pathological processes of the retina. It is also typically seen in senile degeneration of the peripheral retina.[9] In these latter cases the structures of the proliferated perivascular glia undergo hyaline degeneration (fig. 29). More hyaline substance may then become deposited on hyalinized parts of these degenerated cells and large hyaline bodies may develop which can be seen as yellowish white glistening drusen within the periph-

eral retina at ophthalmoscopic examination (fig. 30). The significance of the hypertrophy and proliferation as well of the secondary hyaline degeneration of the perivascular glia is not understood. There is no doubt that this occurrence is nonspecific and may not only be seen in chronic eye diseases but also in senescence.

4. Oligodendroglia

Very little is known about the oligodendroglia of the retina. We were able to demonstrate cells of this type in some cases around ganglion cells of the human retina. However, our findings are not consistent and it seems wise to wait for more complete stains of these cells and their systems in the retina.

5. Microglia

It was already emphasized above that microglia represents the only type of retinal glia that is not neuroectodermal but mesodermal in origin. This is somewhat confusing. However, the term of microglia is used for the same cells in the pathology of the

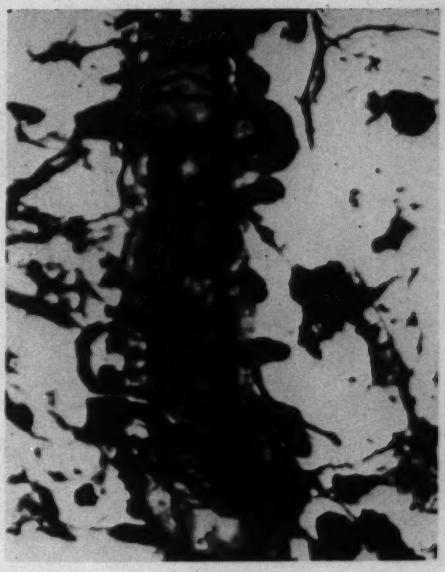


Fig. 27 (Wolter). Hypertrophic perivascular glia coiled around a capillary in the retina of a 72-year-old man with absolute glaucoma. (Frozen section, Hortega stain, photomicrograph.)

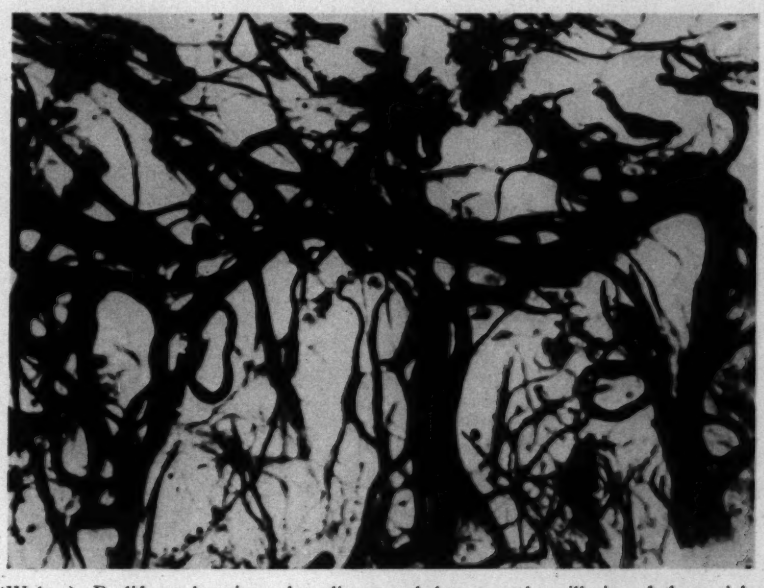


Fig. 28 (Wolter). Proliferated perivascular glia around degenerated capillaries of the peripheral retina of a 75-year-old man with retinitis pigmentosa. (Frozen section, Hortega stain, photomicrograph.)

Fig. 29 (Wolter). Spiderlike formation of degenerating perivascular glia in the peripheral retina of a 70-year-old-man. (Frozen section, Hortega stain, photomicrograph.)

central nervous system and it would not be right to use another term for these cells as they occur in the retina. The microglia are the phagocytes of the retina and it is easy to remember their functions when we recall that they really are the histiocytes of the central nervous system. Microglia normally represent small cells with a round nucleus and a few short branching processes. They are wandering elements and their number will

Fig. 30 (Wolter). Large hyaline bodies in the peripheral retina of a 70-year-old man as an end-result of degeneration and hyalinization of perivascular glia. (Frozen section, Hortega stain, photomicrograph.)

always be found increased when destruction of retinal nervous tissue has occurred. Their main function is phagocytosis. After phagocytosis they are round cells which still have their small nucleus. They are easily detected by a fat stain since the main substances of the destroyed neurons are lipids.

The microglia carries the lipids of the neurons to the blood vessels and thus eliminates the debris of destroyed retinal structures. There are retinal conditions in which the phase of delivery of the lipids from the microglia to the blood is disturbed.[20, 21] The fat-filled microglia (gitter cells) then pile up in the retina, usually in the more loosely arranged macular area or around blood vessels. These cells stay there and finally degenerate. The free lipids remain in the retina and may later become hyalinized. Conditions in which this occurs are hypertensive or diabetic retinopathy which allow for studies of the structure and the phases of degeneration of the retinal microglia.

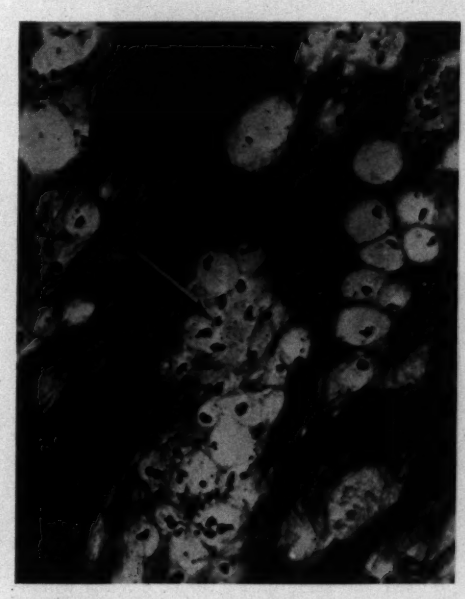


Fig. 31 (Wolter). Flat section through Henle's fiber layer of a retina with advanced hypertensive retinopathy. Many large fat-filled microglia are seen accumulated between the fibers. (Frozen section, Hortega stain, photomicrograph.)

Fig. 32 (Wolter). Flat section through Henle's fiber layer of a retina with more advanced hypertensive retinopathy. Fat-filled microglia, free lipids, and nuclei of hyalinization (arrows) are seen. (Frozen section, Hortega stain, photomicrograph.)

Figure 31 shows accumulation of microglia filled with fat in Henle's fiber layer of the macular area in a case of hypertensive retinopathy. Figure 32 shows a more advanced phase of the same process with microglia, free lipids and areas of hyalinization also from a case of hypertensive retinopathy. Clinically the different stages of accumulation, degeneration, and hyalinization of the microglia in hypertensive or diabetic retinopathy can be seen with the ophthalmoscope and are known as the deep hard exudates and the star figure of the macula area.

6. Elements and changes of the retina not related to retinal glia but may imitate changes of latter

It was described above that retinal astroglia may proliferate on the inner surface of the retina. This should not be confused with the proliferation of fibroblasts on the inner surface of the retina which is a very common occurrence in eye pathology. Proliferation of

Fig. 33 (Wolter). Proliferating fibroblasts on the inner retinal surface in a case of endophthalmitis. Inflammatory cells are seen among the fibroblasts. (Frozen section, Hortega stain, photomicrograph.)

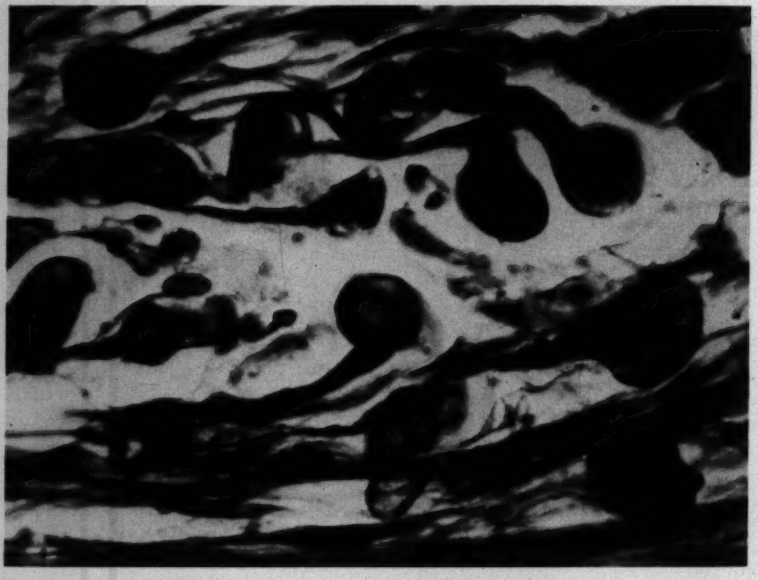


Fig. 34 (Wolter). High-power view of a flat section through the nerve fiber layer of a retina within the area of a so-called cotton wool spot. Terminal swellings of interrupted nerve fibers are seen. All of these point to the right (this is the direction toward the optic disc in the slide). (Frozen section, Hortega stain, photomicrograph.)

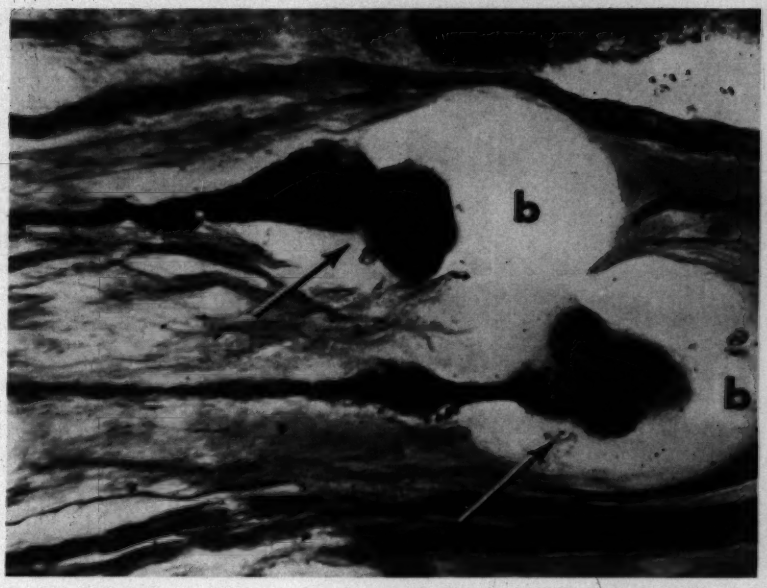


Fig. 35 (Wolter). Two terminal swellings of nerve fibers of the nerve fiber layer of the human retina. It can be seen that the so-called pseudo-nucleus of the cytoid bodies is formed by the terminal swellings of the interrupted nerve fibers (arrows). A zone around these which is not stained by our technique (b) probably represents the pseudo-protoplasm. (Frozen section, Hortega stain, photomicrograph.)

fibroblasts is typically observed in inflammatory involvement of the posterior chamber or in posterior chamber hemorrhages (retinitis proliferans). The fibroblasts originate from the vascular connective tissue of the retina. Figure 33 gives a typical picture of the spindle-shaped and usually branching cells with small oval nuclei. These often surround new-formed thin-walled blood vessels and may also be accompanied by inflammatory cells in cases of inflammatory involvement.

It has been mentioned in the literature that the retinal glia takes part in the formations that we call cotton wool spots after ophthalmoscopic examination. Our studies of such cotton wool spots indicate that retinal glia has no part in these formations.[22] Cotton wool spots are accumulations of numerous terminal swellings of interrupted neurites of the nerve fiber layer usually as a result of local ischemia (fig. 34). All these terminal swellings are at one phase of their development connected with the stump of the interrupted neurite and they all point towards the optic disc. It is interesting to observe that a peculiar zone is found around each terminal swelling of nervous substance (fig. 35). This zone does not stain with the nerve fiber staining methods. It is our impression that the terminal swelling itself represents the central structure that has been called the pseudonucleus of the cytoid bodies while the peripheral unstained zone may be what has been called the pseudoprotoplasm of these structures.

Finally it may be mentioned that a cellular element is long known to be in the vitreous. It has been thought that these cells may be glial in character. We were as yet not successful in adding anything worthwhile to the understanding of the character of these cells. Figure 36 shows an area of a dense zone of vitreous which was found attached to the

Fig. 36 (Wolter). Cellular nuclei in the condensed vitreous of a 70-year-old man. The cell bodies of these nuclei do not stain with the silver carbonate techniques. Bizarre fiber structures are found in the vitreous. (Frozen section, Hortega stain, photomicrograph.)

peripheral retina in the eye of an old man. It shows fiber structures which have an arrangement towards the retina and were found to enter actually into the superficial retinal layers.[9] It also shows cellular nuclei which are oval in shape. The cells of these nuclei do not stain with the silver methods used for the demonstration of the retinal glia. This might be used as a certain evidence against the theory that the cells in the human vitreous are glial cells. However, other methods should be used which show the shape and arrangement of these cells before any final statement is made.

It must be emphasized that this paper is a summary of findings and observations that were made over a number of years about the structure and pathology of the retinal glia. I realize that many of my findings may be incomplete and poorly understood. However, I should like to give this summary to anybody who might want to add to and to correct my conception of the glia of the human retina.

Summary

This is a demonstration of the different types of glia found in the human retina and of their changes under pathologic conditions.

Eye Clinic,
University of Michigan Medical Center.

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Discussion

Parker Heath, M.D. (Sullivan Harbor, Maine): The essayist reports upon further work and summarizing studies of normal and pathologic ocular tissues with special reference to glial cells in the retina. His methods: gross and microscopic study using conventional paraffin embedding, formol fixation, hematoxylin-eosin staining coupled with frozen and brom-formol fixed ocular material and modified Hortega's silver carbonate staining. Some clinical evidence is included and current literature reported.

The essayist finds three main types of neuroectodermal glia: (1) radial fibers of Mueller, (2) astrocytes, with subtypes (lemmocytes), and (3) perivascular glia. Generally the glia seem to supply an intermediate tissue between neurites and blood supply, actually functions are speculative. However, structural alterations with senescence, injury, and disease are observable. Since the glial tissue lies largely in the inner layers of the retina the range and distribution are limited of pathological reactions. Nevertheless many important pathological changes are recognized. Among these are: old age effects upon glial processes, a lessening in number and a coarsening of surviving fibers, cystic degenerations, hyalinization and formation of hyaline bodies, glial scarring and diverse proliferations. The proliferation of glial cells upon the internal limiting membrane of the retina is an especially interesting nonspecific activity.

The essayist has found by his method that Mueller's fibers are relatively passive compared with astroglia. Cogan and Kuwabara* have recently found by use of tetrazolium and lactate-DPN substrate considerable dehydrogenase activity in Mueller's fibers and both glial and ganglion cells. Dr. Wolter has found advanced degenerations and scarring stimulate band and rosette formations which may derive from Mueller's fibers. The relationships of the rosettes and those of the peripheral retina to secondary vitreous will bear further study.

The perivascular glia offers considerable speculative possibility as to function. Degenerations and hypertrophies of these cells under different pathologic influences are of great interest.

The author has limited the origin of "cotton wool" spots as seen with the ophthalmoscope to terminal swellings of interrupted neurites of the nerve fiber layer. This excites some disagreement. Usually collections of lipophages are considered to be a principle source of the spots. No doubt the interrupted neurites are one form of cytoid body.

Proliferative activities in the peripheral retina and vitreous, possibly not glial, is an important observation and likely to call for further study and evaluation. Inter-action of glia and fibrocytes are mentioned in scar formations. Incidently the massive proliferative scars sometimes seen with retinal hemorrhages in children are nonspecific reactions which should be called Coats' reaction and not "disease." Mention is made of the relative failure to stain retinal oliogodendroglia.

In conclusion the affinity of nervous tissue and especially glial cells for the stain is so marked that some reeducation is necessary in making interpretations. The contrasty effects sometimes are achieved at the expense of gradation and detail. Whatever the limitations, the method makes it possible to form a fresh imagery in areas which formerly were obscure, poorly stained tangles. By this method identification of the frame-work of the inner layers of the retina becomes possible.

The difficulties of precise interpretation are substantial because by the method unknowns became compounded with knowns Reactivity is mostly nonspecific. We must at this time hang our interpretations largely upon morphology. In due time as data accumulate from other methods, such as those which elucidate metabolic cycles, then physiologic and morphologic observations will give us well-rounded knowledge of the retina.

* Cogan, D. G., and Kuwabara, T.: Retinal dehydrogenases. Tr. Am. Ophth. Soc., 1959, in press.

THE ROLE OF INSULIN IN LENS METABOLISM*

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Cleveland, Ohio
and
J. W. Patterson
Nashville, Tennessee

The uptake of glucose by rat lenses incubated in Tyrode's solution containing 100 mg. of sugar per 100 cc. is significantly increased if insulin is injected intravenously prior to removal of the lens.[1,2] The results are the same whether the uptake of glucose is measured as glucose disappearing from the medium or as glucose disappearing from the total system of lens plus medium.[3] On the other hand, the addition of insulin directly to the incubation medium does not increase the uptake of glucose by lenses obtained from untreated animals.[2-4]

These facts suggest either (a) that insulin is ineffective in the form injected and is activated in some way following injection, (b) that in order to be effective insulin requires the presence of one or more additional substances or (c) that insulin does not act directly on the lens, but rather stimulates the production or release of some other substance which is transported to the lens and affects lens metabolism.

Each of these possibilities implies that some organ other than the lens is necessary if the action of insulin on lens metabolism is to be demonstrated. It is the purpose of this paper to report the results of experiments designed to determine which organ or organs may be required to produce an increase in glucose uptake following the injection of insulin.

* We wish to express our appreciation for the support received from the National Institute of Arthritis and Metabolic Diseases, of the National Institutes of Health, U. S. Public Health Service Grant Number A-154 and for a fellowship from the National Council to Combat Blindness, New York City, in support of one of the authors (T. G. Farkas).

† In the totally eviscerated rats the external jugular was used.

Experimental

Male Sprague-Dawley rats weighing 120-170 gm. were anesthetized by the intravenous injection of 6.6 mg. Nembutal/kg. body weight. In each series of experiments the animals were divided into two major groups. Sham operations were performed on a control group and certain organs were removed from the experimental group. Immediately following operation all animals were injected subcutaneously with 1.0 ml. of a 30-percent glucose solution. At the same time half of each group received 20 units of insulin by injection into the surgically exposed inferior vena cava.† One hour after this treatment the animals were decapitated and the lenses were removed and placed in Tyrode's solution containing 100 mg.% of glucose. The glucose uptake was determined as previously described by measuring glucose disappearance from the medium[2] using the Somogyi method.[5] The figures given on the bar graphs are the average values and standard deviations.

Results

The data are presented in Figures 1 through 6 and the following points may be emphasized:

1. The values for glucose uptake by lenses from sham-operated animals, both with and without insulin injection (fig. 1-6), are in good agreement with those previously reported for normal animals.[1] The increase in glucose uptake observed following the injection of insulin has been termed the "insulin effect."

2. Total evisceration, that is the removal of the stomach, intestines, spleen, pancreas, kidneys, adrenals and liver, abolishes the "insulin effect" (fig. 1).

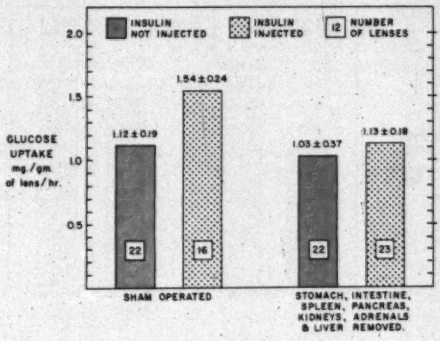

Fig. 1 (Farkas, et al.). Effect of evisceration on glucose uptake of rat lenses.

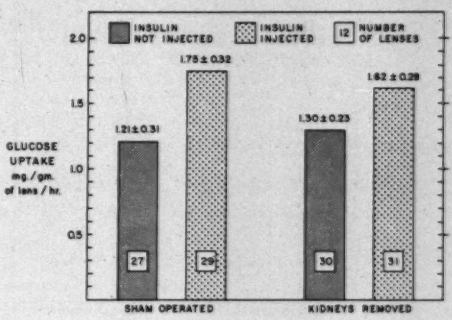

Fig. 3 (Farkas, et al.) Effect of nephrectomy on glucose uptake of rat lenses.

3. Removal of the stomach, intestines, spleen, pancreas, (fig. 2) and in a separate series of experiments the kidneys (fig. 3), does not modify the "insulin effect."

4. Removal of the liver along with the stomach, intestines, spleen and pancreas produces different results, for, under these circumstances the "insulin effect" is abolished, as it is following complete evisceration (fig. 4).

5. Following adrenalectomy the glucose uptake of the lens is increased to the level obtained in normal animals following the injection of insulin (fig. 5). This may be termed the "adrenal effect." The injection of insulin in the adrenalectomized animal produces no further increase in the uptake of glucose (fig. 5). Removal of the stomach, intestines, spleen and pancreas along with the adrenals does not modify the "adrenal effect" (fig. 6).

Discussion

It is apparent that the adrenals and the liver are the key organs involved in explaining the results obtained. The stomach, intestines, spleen, pancreas and kidneys do not modify either the "insulin effect" or the "adrenal effect" and can be eliminated from consideration. Stimulation of the glucose uptake of the lens, as observed after adrenalectomy or the injection of insulin, can be demonstrated only when the liver is intact, indicating that the liver plays a stimulatory role. Conversely, since removal of the

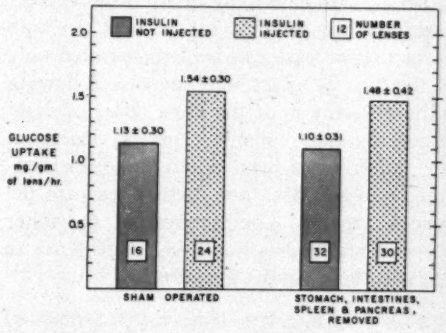

Fig. 2 (Farkas, et al.). Effect of removal of the stomach, intestines, spleen, and pancreas on glucose uptake of rat lenses.

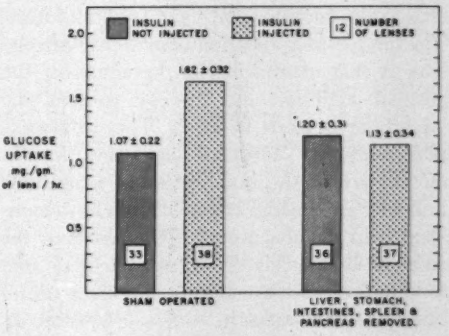

Fig. 4 (Farkas, et al.). Effect of removal of the liver, stomach, intestines, spleen, and pancreas on glucose uptake of rat lenses.

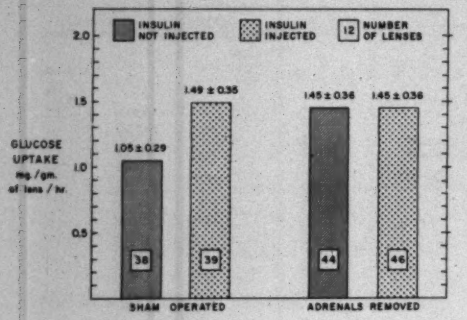

Fig. 5 (Farkas, et al.) Effect of adrenalectomy on glucose uptake of rat lenses.

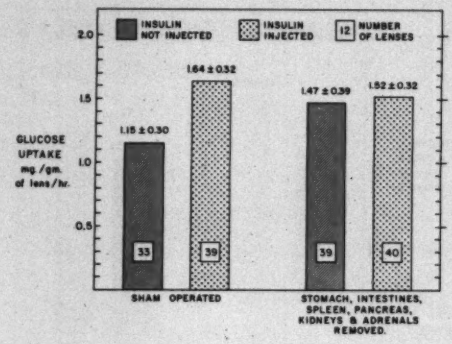

Fig. 6 (Farkas, et al.). Effect of removal of the adrenals, stomach, intestines, spleen and pancreas on glucose uptake of rat lenses.

adrenals is accompanied by an increase in glucose uptake, its influence in the intact animal must be inhibitory.

The adrenals could exert their effect by secreting an inhibitor which either acts directly on the lens or prevents the formation or action of the liver stimulator. Although, as noted, removal of the liver along with the adrenals abolishes the "adrenal effect," removal of the liver from an animal with intact adrenals does not decrease the glucose uptake below the normal level. This finding suggests that the adrenal inhibitor and liver stimulator do not act independently on the lens but rather that the adrenal inhibitor either blocks the production of the liver stimulator or neutralizes its action during or after secretion.

The "insulin effect" can be demonstrated only if the adrenals and liver are both intact. The simplest explanation consistent with the data is that insulin blocks the action of the adrenal inhibitor and thereby permits the stimulator to exert its effect. This would explain why the "adrenal effect" and "insulin effect" are of the same order of magnitude and why an "insulin effect" cannot be demonstrated in the absence of the adrenals or the liver. Alternatively, it is possible (a) that insulin increases the secretion of liver stimulator or potentiates its action, (b) that insulin stimulates the liver to produce another substance which by itself or acting synergistically with insulin stimulates the lens, or (c) that the liver modifies the injected insulin and thereby makes it effective. These alternatives, however, require the additional assumption that after adrenalectomy the enzyme systems of the lens are operating maximally and cannot be stimulated further by insulin injection.

The results suggest comparisons with the results obtained in the whole animal in which the blood sugar can be lowered either by the injection of insulin or by removal of the adrenal glands. The present findings may contribute to a localization of this effect.

Summary

Studies have shown that the glucose uptake of isolated rat lenses can be increased either by adrenalectomy or by insulin injection prior to removal of the lens. In both cases the increase can be demonstrated only if the liver is intact. The increase following adrenalectomy is of the same order of magnitude as that following insulin injection, and injection of insulin into an adrenalectomized animal does not further increase the glucose uptake. The removal of the other visceral organs has no effect. Hypotheses to explain these results are offered.

Western Reserve University School of Medicine (6).
Vanderbilt University School of Medicine (5).

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Discussion

Dr. John F. R. Kuck, Jr. (Detroit): The essayists are to be commended for the progress made in implicating the liver and adrenals with insulin action and lens glucose metabolism. It is particularly wise, I think, to avoid using diabetic animals as long as information can be obtained otherwise, since severe diabetes brings so many complications that secondary effects on the lens may mask any primary fault in glucose metabolism.

The present experiments demonstrate clearly that in the presence of an intact liver either insulin injection or adrenalectomy will increase lens glucose uptake. This effect occurs after glucose injection.

I would like to ask if the glucose injection is necessary to show the phenomenon, and what effect this 300 mg. of glucose has on the blood sugar level and the lens glucose level. If the rise in blood glucose is appreciable, then it is possible that the intact liver serves merely as a converter-storage system for the removal of excess glucose and its storage as glycogen. This system, of course, is inoperative after hepatectomy.

Dr. T. G. Farkas (closing): Glucose was injected into our animals after operation to be able to maintain them alive for one hour, especially after hepatectomy.

As we are all aware, the blood glucose level drops rather precipitously after hepatectomy, and the animals could not be kept alive for the duration of the experiment without the supplement of glucose.

Since the sham-operated animals were also injected with the same amount of glucose, and the glucose uptake in these lenses was exactly the same as in normal animals in which glucose was not injected, I do not think the injected amount of glucose plays any role in the phenomenon we did observe.

ULTRASTRUCTURE OF THE IRIS: AN ELECTRON MICROSCOPIC STUDY*

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The electron microscope with its manyfold increase in resolving power over the light microscope and several improved techniques of specimen preparation have been applied to the study of ocular tissues. Our main interest has been focused on the mammalian iris, the histology of which has heretofore been based almost entirely on the use of conventional techniques.[1-3] The inherent limitations of the latter together with new ideas derived from other studies have introduced a number of questions concerning the finer structure of this tissue:

a. Is there a continuous cellular border layer on the anterior surface of the iris?

b. Are there specialized endothelial cells on this surface?

c. What is the nature of the "thick walls" of the stromal vessels?

d. What is the nature of the abundant ground substance of the iris stroma?

e. What is the relationship of the pigment epithelium to the dilator muscle?

f. Is there an internal limiting membrane on the posterior surface of the iris compara-

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† Dr. Fine is a special trainee of the National Institute of Neurological Diseases and Blindness, NIH.

ble to that which has been observed to invest the ciliary epithelium?

These are some of the questions we hoped to answer, at least in part, when our studies were begun.

Materials and Methods

Irises for this study were obtained from the eyes of rhesus monkeys and man. Eyes from young (18 months) rhesus monkeys were enucleated within five minutes of death by exsanguination or under anesthesia before death. The anterior segment was removed by making an incision behind the pars plana with a sharp razor blade and completing the circumferential incision with scissors. The lens and anterior vitreous body were removed by grasping the lens with forceps and severing the zonules with scissors. The resulting "cup" consisting of the iris, ciliary body and cornea was cut into quarters. Each quarter was then placed in stoppered test tubes containing cold (0°C.-4°C.) one-percent osmium tetroxide[4] in isotonic sodium acetate veronal buffer of pH 7.35. Fixation time was three hours unless stated otherwise. A modification of this procedure was to first replace the aqueous with the fixative, and then to proceed in the manner just described. After fixation the tissue was washed in cold isotonic veronal buffer (pH 7.35) and distilled water. Dehydration was carried out in cold graded ethanol (30, 50, 70, 90 percent, and absolute) with changes every 15 to 20 minutes. After an overnight (14-16 hours) storage in cold 50-50 ethanol and methacrylate monomer, embedding was carried out in methacrylate[5] by placing the tissue for one hour in 20/80 parts by volume methyl/butyl methacrylate followed by two changes, 30 minutes each, of the same mixture of monomers containing one-percent 2,4-dichlorobenzoyl peroxide with dibutyl phthalate (Luperco). The tissue was oriented for sectioning tangential to the pupil as well as radial in prepolymerized methacrylate. Polymerization was completed in an oven at 65°C. for four hours.

The normal human eye used in these studies was obtained from a 42-year-old Negro man who underwent surgery for a maxillary carcinoma, which had extended into the left orbit. Preoperative clinical examination showed the eye to be normal upon tonometric, slit lamp and ophthalmoscopic examinations. This eye was removed together with the orbital contents. Within 30 minutes after removal, the anterior chamber was irrigated with one-percent osmium tetroxide and treated in a manner similar to that described for the rhesus monkey.

Sections of the methacrylate embedded tissues were made with a glass knife in a Porter-Blum type[6] ultramicrotome. Thickness of sections as given by the approximate calibration of the instrument or judged from interference colors were as follows: 0.5 to 2.0 micron-thick sections were stained for light microscopy after removing the methacrylate with xylene and hydration in graded (decreasing concentration) ethanol. Ultrathin sections appearing colorless, silver or gold on the knife trough containing 10-percent acetone in water were "spread" with chloroform or xylene fumes[7,8] and placed on carbon coated[9] uniformly thin films of formvar.[10]

In addition to these methods for the study of iris structure, we have employed such other techniques as shadowcasting of "thick sections" (0.1 micron), differential centrifugation of components followed by electron microscopy and histochemical studies using ultrathin sections. The results of these investigations will be reported separately.

An RCA, EMU-3B instrument utilizing a 40-micron platinum objective aperture and a 10-mil Canalco externally adjustable condenser aperture was used. Electron micrographs were made at electron magnifications of 1,600 through 15,000 diameters.

Results

The observations to be recorded are applicable to both man and rhesus monkey except where otherwise stated.

The anterior surface of the iris was found to consist of aggregates of both pigmented and nonpigmented cells with their cytoplasmic processes extending over portions of the surface (figs. 1-3, 5-8). Morphologically these surface-forming cells were identical to all other pigmented and non-pigmented stromal cells (figs. 7, 10 and 11). No specialized surface endothelium could be identified.

Apertures varying in width from a fraction of a micron up to ten microns or more were observed between the cell aggregates on the surface. For the examination of larger apertures use was made of the light microscope.*

Throughout the stroma, collagen fibrils of varying lengths, approximately 600 Å in width, and with 500-600 Å axial periodicities were observed in increasing concentration towards the cells or aggregates of cells, blood vessels and nerve fibers. In the remaining "spaces" of the stroma the presence of other materials, in addition to that surrounding the blood vessels (figs. 17 and 18) could not be demonstrated with the methods used in this study.

Pigmented cells from the monkey iris stroma contained in their cytoplasm elongated pigment granules oriented parallel to the major axis of the cell or its extensions, while the pigment granules of the human stroma cells were short ovoids[13] without apparent orientation (figs. 9 and 12). The pigment epithelium of both monkey and man contained granules larger in size than those of the stroma. These granules were similar in size and shape in the two species (figs. 28 and 29).

Clump cells were found only in the human and near the sphincter muscle. They were much larger than the other pigmented stromal cells containing numerous aggregates of pigment granules (figs. 13 and 14). They appeared to be more uniform in shape, rounded without any large protoplasmic extensions. Fine cytoplasmic processes (about 0.1 micron wide) were present on their surface.

Figures 15 through 18 show blood vessels found in the iris stroma. In the monkey iris, vessels were found in all portions of the stroma, including the anterior border layer. The iris vessels observed in this study consisted of a single layer of endothelial cells; their internal diameter varied from just under one to about ten red blood cell diameters. Frequently the adjacent stromal cells were very closely applied to these vessels (fig. 17). An amorphous material sometimes appearing fibrillar, occupied the spaces between the vessel endothelium and adjacent cells.

Groups of myelinated and nonmyelinated nerve fibers were found throughout the stroma of both monkey and human irides (figs. 19 and 20). At the iris root the nerves appeared to be more closely grouped (fig. 19). The axoplasm of both myelinated and nonmyelinated nerve fibers contained mitochondria with the typical internal membranes. In addition, filaments, measuring about 100 Å in width, were present in the axoplasm (fig. 21). A Schwann cell is shown in Figure 22.

The sphincter muscle consisted of bundles (groups) of smooth muscle cells oriented, for the most part, parallel to the pupillary margin (figs. 23 and 24). Large numbers of mitochondria were aggregated axially in these cells. Pigment granules were occasionally observed in these muscle cells.

The dilator muscle consisted of a few layers of elongated smooth muscle cells, loosely interdigitated (figs. 25 and 26). They were closely applied to the adjacent pigment epithelium and frequently contained pigment granules. These granules were similar in size and shape to those of the adjacent pigment

* Gregersen[11, 12] has carried out perfusion experiments with dextran and killed bacteria on human and rabbit eyes. He observed that these macromolecules (less than 1.0 μ in diameter) diffused into the iris not only through the crypts observable by light microscopy but also through "the more solid parts of the anterior surface." Our own studies of the ultrastructure of the iris provide an anatomic explanation for Gregersen's experimental observations and support his conclusions.

epithelium. Cytoplasmic continuity into the muscle layer of this pigment epithelium was observed (fig. 25). Between the muscle cells a homogeneous material of similar electron opacity to that found about the blood vessels was seen. Mitochondria were present in large numbers in these muscle cells.

The pigment epithelium consisted of essentially two layers of pigmented cells (figs. 27-29). Intercellular spaces containing microvilli were observed between the anterior and posterior layers. The intercellular spaces between adjacent cells within the anterior and posterior layers of epithelium rarely contained these microvilli. The free surface of the posterior layer of epithelium was limited by a membrane-like structure. This "membrane" frequently appeared to consist of several layers (fig. 28), and extended over the entire posterior surface of the iris from root to pupil. Infoldings of the cell membrane of the posterior surface, similar in appearance to those described in the inner cells of the ciliary epithelium were found (figs. 14, 15, 16).

Conclusion

The results of our electron microscopic studies of the human and rhesus monkey iris have been in general agreement with observations based on conventional histologic technique. We have, however, been able to add a more detailed description, heretofore lacking, of several important structures.

With regard to the controversial issue as to whether a specialized endothelial anterior surface layer exists, our observations have failed to provide any evidence of the presence of such a structure. The cells present on the anterior surface of the iris appeared to be identical with the pigment-containing and nonpigmented stromal cells. These cells, however, do not form a continuous cellular covering and there are openings into the iris stroma measuring from less than one micron to at least ten microns.

The thick adventitial tunic which has long been known to characterize the iris vessels consists of typical collagen fibrils identical with those found throughout the iris stroma. All of the iris blood vessels we have observed, presented structural characteristics of capillaries even though their lumens were often very large.

The nerves found in the iris stroma included both myelinated and nonmyelinated fibers.

The anterior and posterior layers of iris epithelium are separated by spaces containing microvilli which project from the epithelial cells. Numerous intercellular spaces were found between the cells of the posterior epithelial layer. The free surface of the latter was covered by a membrane-like structure which in the monkey was often laminated and covering the entire posterior surface.

Armed Forces Institute of Pathology (25).

Acknowledgment

We wish to thank Dr. Lorenz E. Zimmerman, Chief, Ophthalmic Pathology Branch, Armed Forces Institute of Pathology, for his suggestions and guidance during the preparation of this manuscript and Dr. Benjamin Rones, director, Ophthalmic Pathology Laboratory, Washington Hospital Center, for his interest and efforts on behalf of this project. We also express our appreciation for the technical assistance of Aubrey C. Jenkins.

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Discussion

George K. Smelser, Ph.D. (New York): Introduction of the electron microscope has allowed the study of problems which have intrigued anatomists and ophthalmologists for many years. Answers to such problems often provide us with new questions. It would seem that we are insatiable in our efforts to understand the structure of the eye, and it is this characteristic which I offer as an excuse for the form of my discussion, which must consist in the asking of more questions. The absence of a complete epithelial covering of the iris has long been suspected in man, and debated. The present paper reports that there are holes of considerable magnitude in the surface epithelium. What is the shape of these holes, and how may they be affected in their size or shape by fixation or post-mortem changes? As the resolution of our microscopy increases, the difficulty in avoiding artifacts increases with it. One must be ever alert to the possibility that the structures we see under high magnification do not necessarily represent those which were in existence before death. This is a problem to all electron microscopists, and not solely a problem confronting the present authors. Connective tissue, which appears in these photomicrographs to be bathed in aqueous humor, has usually been thought to be embedded in a gelatinous ground substance.

According to the structure of the iris as described in this paper, the ground substance of the connective tissue would be in contact with aqueous humor. Is there any evidence of the existence of such material surrounding the capillaries and collagenous fibers in the iris?

A solution to an intriguing question may lie in the hands of Doctors Tousimis and Fine. The origin of the "clump cells" has long been debated, some authors believing them to be derived from pigment epithelium, others regarding them as connective tissue derivatives. Since differences in the shape of the pigment granules of the epithelial and connective tissue cells have been described in this paper, perhaps an answer to this question is at hand. Have you evidence that the clump cells are epithelial in origin? The authors describe intercellular spaces lying between the two epithelial cell layers of the iris in which delicate cytoplasmic villi extend. I cannot believe that such villi are present without function. Would the authors comment on this?

In our investigations of the same epithelial layers in the ciliary body, Dr. Pappas and I described desmosomes, or intercellular bridges, which serve to attach the two epithelial layers together. Were no such "bridges" seen? A most interesting observation has been presented—that of infoldings of the cellular membrane of the iris epithelium where it is exposed to the posterior chamber. Similar infoldings were described in such cells on the ciliary processes. These infoldings have been thought important in relationship to formation of aqueous humor by Dr. Holmberg, and Dr. Pappas and myself. Is there any evidence that these somewhat similar modifications of the cellular membrane have some functional significance with reference to aqueous humor formation or resorption? It would be interesting to hear the authors' comments on this point.

It may be that some of you have the impression that my many questions arise from some dissatisfaction with this paper. I assure you that such is not the case. The questions arise because of stimulation that this report has given, because it has whetted the appetite for more information on the structure of the iris.

A. J. Tousimis (closing): Thank you, Dr. Smelser, for your excellent discussion of our paper.

An attempt will be made to answer some of your questions and to remark on others.

The post-mortem effects before fixation, on cell ultrastructure, has been observed by many investigators within the past few years. This constitutes one of the biggest problems in the application of electron microscopy to the study of pathologic material. It appears, from already published work, that for preservation of all accepted structural detail the tissue should be fixed within 10 minutes. For most

of our work—in fact, for all the rhesus monkey eyes—we have fixed the iris within five minutes of enucleation. Thus structural changes, as described by others for tissues, for example, intestinal mucosa, fixed at longer periods after removal of tissue, have not been observed.

Whether post-mortem effects alter the anterior surface of the iris within this limited time, we do not know. However, in experiments designed to test for these effects—irises fixed up to one-half hour after enucleation did not show any changes on the anterior surface.

A material of higher electron density in the intercellular spaces of the iris pigment epithelium has been seen. The question regarding the chemical identification of this substance might possibly be answered with experiments utilizing suitable electron stains coupled with the appropriate electron microscopical techniques.

The origin of the clump cell in the human iris stroma is an interesting question. However, any attempt to answer it on the structural evidence alone, should be made very cautiously. Pigment granules found in these cells resemble both those of the stromal and of the pigment epithelial cells. Even though microvilli, similar to those of the pigment epithelium, have been seen on these cells, cytoplasmic extensions similar to those of either pigmented or nonpigmented cells were not found. On this evidence alone, therefore, one could not establish the origin of this cell.

The functional significance of the microvilli of cells forming the pigment epithelial layer of the iris is another important comment by Dr. Smelser. We have not conducted studies aimed to answer this question. At this time, therefore, one can only refer to ultrastructural studies of other cells showing microvilli with known physiologic functions (that is, secretory).

Desmosomes have been described in many electron-microscopic studies of epithelial tissues. We have observed the cytomembranes of the pigment epithelium cells approaching very closely to one another, yet we have not seen any desmosomes.

Finally, in regard to the question concerning the cytomembrane-infoldings on the posterior surface of the pigment epithelium, one may rely again on suggested functional findings by others, that is, secretory, on tissue composed of cells with similar structure. Thank you.

EXPLANATION OF FIGURES (Tousimis and Fine)

Fig. 1. Radial section near the pupillary margin of the rhesus monkey iris. The sphincter of the pupil is seen in cross-section consisting of bundles of smooth muscle cells. The dilator muscle is not present. In the stroma pigmented and nonpigmented cells, capillaries and nerves are found. (OsO_4 fixation. Methacrylate embedding, 1.5 micron thick section, Giemsa stain, ×305.)

Fig. 2. Section tangential to the pupil (rhesus monkey) near the pupillary margin showing the sphincter muscle cut longitudinally. The nuclei are cut along their major axes indicating the general direction of the muscle fibers. The numerous blood vessels in cross-section appear throughout the stroma and between the sphincter and pigment epithelium. The anterior surface appears similar to that observed in the radial sections. (OsO_4 fixation, methacrylate embedding, 1.5 micron thick, Giemsa stain, ×305.)

Fig. 3. A higher magnification photomicrograph, 1.5 micron thick, radial section, of the anterior surface of the rhesus monkey iris. Blood vessels are observed frequently as shown here near the surface. Aggregates of both pigmented and nonpigmented cells surround a blood vessel (capillary) at the surface. Another blood vessel, with a single endothelial lining occupies the lower center of the field. Openings on the surface are readily seen between the stromal cells. (OsO_4 fixed, methacrylate embedded. Giemsa stain, ×820.)

Fig. 4. A double-layer of pigmented cells limit the posterior surface of the monkey iris. The cytoplasm of these cells contain large numbers of pigment granules. The dilator muscle is closely approximated to the anterior surface of these pigmented cells. In the adjoining stroma both pigmented and nonpigmented stromal cells are seen together with a nearby capillary. (OsO_4 fixation, methacrylate, 1.5 micron thick, Giemsa stain, ×820.)

Fig. 5. A low magnification electron micrograph of the anterior iris surface (rhesus monkey). This surface is formed by both pigmented and nonpigmented cells. These cells appear to be morphologically identical to those throughout the stroma. Apertures, ranging between 1.0 and 2.0 microns in width are present. Long narrow protoplasmic extensions of the nonpigmented cells extend over nearby pigmented cells. Pigmented cells are also exposed on the surface (×2,650).

Fig. 6 (Tousimis and Fine). Electron micrograph of rhesus monkey iris surface, showing some of the pigmented cells in longitudinal and others in cross-section. An aperture is clearly seen on the left. The nucleus of the nonpigmented cell on the surface (NP) is highly indented (×2,650).

Fig. 7 (Tousimis and Fine). A higher magnification of the surface in the region of an aperture (AP). Nuclei of both pigmented and nonpigmented cells are indented. The pigment granules are present throughout the cytoplasm, including the extensions of the pigmented cells. Protoplasmic extensions lacking pigment granules appear both in longitudinal and cross-section (×5,470).

Fig. 8 (Tousimis and Fine). Electron micrograph of anterior iris surface of a brown human iris. Apertures ranging in width from one up to 10 microns can be seen. Pigmented (P) and nonpigmented (NP) cells are found on the surface. These cells are morphologically similar to those found throughout the stroma. The pigment granules differ in size and shape from those of the rhesus monkey. An indented nucleus of a nonpigmented cell (NP) is seen near the center of the micrograph (×2,650).

Fig. 9 (Tousimis and Fine). Pigmented stromal cells (rhesus monkey). Cigar-shaped pigment granules are cut in cross-section in the lower cell (P_2) and the majority are cut longitudinally in the upper cell (P_1). Portions of nonpigmented protoplasmic extensions are seen in the vicinity of the pigmented cells. Beyond these a few nerve fibers (NF) are present (×5,460).

Fig. 10 (Tousimis and Fine). The cytoplasm of a pigmented stromal cell in the rhesus monkey iris contains the cigar-shaped pigment granules, cut in a number of planes. Mitochondria (M) are present in the cytoplasm between the pigment granules. Nonpigmented protoplasmic extensions containing mitochondria lie adjacent to the pigmented cell. Collagen fibrils (CF) cut mostly in cross-section are present in the intercellular areas (×14,580).

Fig. 11 (Tousimis and Fine). Nonpigmented stromal cell of the rhesus monkey iris. The nucleus is indented. Mitochondria (M) and other cytoplasmic components are present in the cytoplasm of this cell. A nerve fiber (indicated by arrows) shows the mitochondria (M_2) and the filamentous composition of its axoplasm. Intercellular collagen fibrils are present (×12,270).

Fig. 12 (Tousimis and Fine). Electron micrograph of a nonpigmented (NP) stromal cell of brown human iris. Mitochondria (M) and small portions of α-cytomembranes (α) (endoplasmic reticulum) are present in the cytoplasm. Portions of the cytoplasm of pigmented cells (P) are seen on either side. These granules differ both in size and shape from those of the rhesus monkey stroma (×14,580).

Fig. 13 (Tousimis and Fine). Electron micrograph of a "clump" cell near the sphincter muscle of the brown human iris. The nucleus is not present in this section. The cytoplasm is filled with many aggregates of pigment granules which appear to vary greatly in size and shape. Only very fine protoplasmic extensions are present on the cell surface (PE) (×7,290).

Fig. 14 (Tousimis and Fine). A higher magnification of a "clump" cell (CC) with its aggregates of pigment granules, mitochondria (M) and some α-cytomembranes (α) (endoplasmic reticulum). The nearby nucleus is that of a nonpigmented stromal cell. A portion of the cytoplasm of a typical pigmented stromal cell (P) is seen in the upper right corner of the micrograph (×13,250).

Fig. 15 (Tousimis and Fine). Electron micrograph of rhesus monkey iris cut tangential to the pupil in the sphincter muscle region. A portion of the sphincter muscle (SM) is seen below. Three blood vessels, one containing a red blood cell (RBC) lie anterior to the muscle. Pigmented (P), nonpigmented (NP) cells and collagen fibrils (CF) are distributed throughout the stroma (×2,175).

Fig. 16 (Tousimis and Fine). A higher magnification of one of the stromal vessels shown in Figure 15 with adjacent stromal cells (P, NP) collagen fibrils (CF) and a somewhat homogeneous material of intermediate electron opacity (BM) (basement membrane) surrounding the endothelial (E) lining. A portion of sectioned red blood cell (RBC) is seen suspended in the plasma (PL) occupying the lumen (×6,375).

Fig. 17 (Tousimis and Fine). Electron micrograph of a sectioned stromal vessel from a brown human iris. The vessel wall is composed of single endothelial cell layer. In close proximity to the outer surface is a quantity of material (BM) of intermediate electron opacity (basement membrane?). Cytoplasmic processes of pigmented stromal cells (PC) are in close apposition (×6,250).

Fig. 18 (Tousimis and Fine). Stromal vessel of the human brown iris in cross-section with its endothelial wall. The nucleus of an endothelial cell is indented (N). Mitochondria (M) are present in the endothelial cell cytoplasmic processes. The material (BM) surrounding the capillary is not uniformly distributed around the vessel. Portions of pigmented stromal cells are in close contact with this material. Collagen fibrils are seen distributed in the adjacent stroma (×6,375).

Fig. 19 (Tousimis and Fine). Electron micrograph of a bundle of nerve fibers near the root of the rhesus monkey iris. Myelinated fibers (MF), Schwann cells (SC) and protoplasmic extensions (PE) of adjacent stromal cells are present (×4,785).

Fig. 20 (Tousimis and Fine). A group of both myelinated (MF) and nonmyelinated (N-MF) nerve fibers in cross-section from the pupillary area of rhesus monkey iris. The axoplasm (Ax) of both myelinated and non-myelinated fibers contain small mitochondria (Ax-M) and filaments (Ax-F). (×10,550).

Fig. 21 (Tousimis and Fine). A cross-sectioned myelinated nerve fiber showing the laminated myelin surrounding the axoplasm. Small mitochondria (Ax-M) and filaments (Ax-F) are present in the axoplasm. Larger mitochondria (M) are present in the surrounding cytoplasm of a Schwann cell (×33,180).

Fig. 22 (Tousimis and Fine). A Schwann cell with its nucleus (N) and myelinated nerve in its cytoplasm. Mitochondria (Ax-M) and filaments (Ax-F) are present in the axoplasm. Larger mitochondria (M) are seen in the cytoplasm of the cell (×33,180).

Fig. 23 (Tousimis and Fine). Pigment epithelium and sphincter muscle (SM) of rhesus monkey iris at the pupil. Sphincter muscle cells appear in cross-section. A small part of the sphincter muscle is exposed at the pupillary area (PA). Numerous mitochondria (M) are present in the cells of the sphincter muscle with a tendency to concentrate axially. The pigment epithelium cells appear elongated near the termination of the pigment epithelium at the pupil. The intercellular spaces (ISp) are prominent. PC = posterior chamber (×4,500).

Fig. 24 (Tousimis and Fine). Electron micrograph of sphincter muscle of rhesus monkey iris cut tangential to the pupil (longitudinal section of the muscle). A pigmented stromal cell (PC) is closely apposed to the anterior surface of the sphincter. The muscle cells contain numerous mitochondria (M) and a filamentous-like cytoplasmic constituent. A less electron-dense material occupies some of the intercellular spaces (ISp) (×6,500).

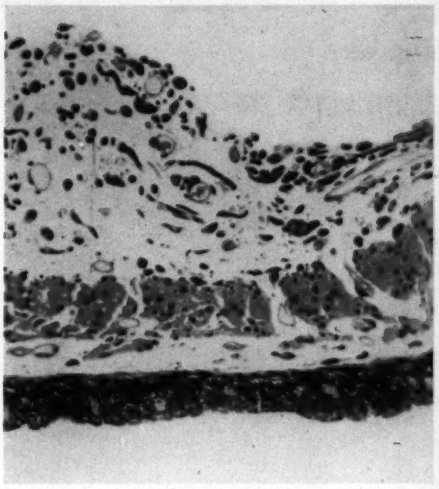
Fig. 25 (Tousimis and Fine). Electron micrograph of the dilator muscle in a radial section of monkey iris. The elongated muscle cells (MC) are loosely interdigitated. A somewhat electron opaque material occupies the intercellular spaces (ISp). Mitochondria (M) are present within the muscle cells in addition to occasional pigment granules (PG). A filamentous material (FM) is present in the cytoplasm of the muscle cells. Direct cytoplasmic continuity of one of the pigment epithelium cells with the muscle layer can be seen (×12,270).

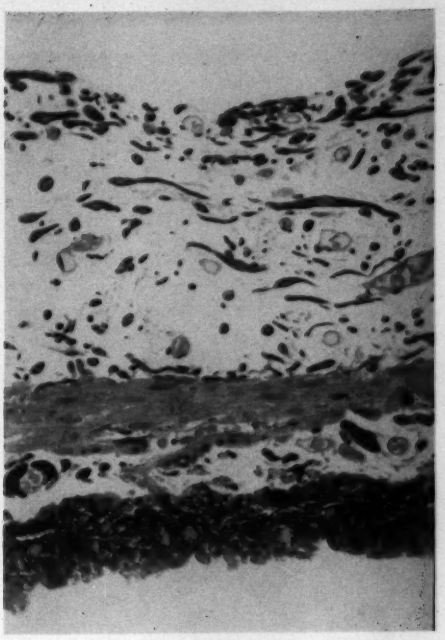
Fig. 26 (Tousimis and Fine). Dilator and pigment epithelium cells are in close apposition to cytoplasmic processes of nonpigmented stroma (ST) cells. The dilator muscle-cell cytoplasm contains a few pigment granules (PG). Intercellular spaces (ISp) containing microvilli are seen between the two layers of pigment epithelium cells. Mitochondria are present in all cells in addition to the filamentous (F) material in the muscle cell (×12,270).

Fig. 27 (Tousimis and Fine). Electron micrograph showing the boundary between the two layers of the pigment epithelium. Pigment granules (PG) are distributed in the cytoplasm of these cells. Mitochondria (M) and other cytoplasmic constituents are also found in their cytoplasm. Microvilli (MV) from the cells project into the intercellular spaces (ISp). Intercellular spaces without microvilli are more prominent between cells forming the posterior layer (rhesus monkey iris) (×7,290).

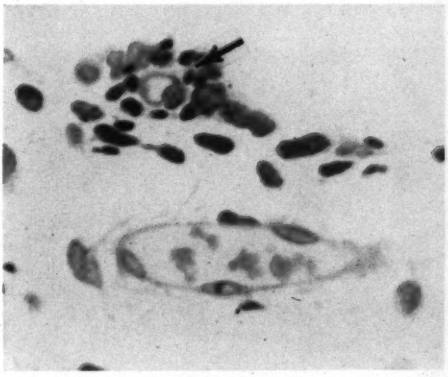
Fig. 28 (Tousimis and Fine). The posterior surface of the rhesus monkey iris is formed by a layer of packed pigmented cells. Intercellular spaces (ISp) are continuous from the dilator muscle layer or anterior layer of pigment epithelium to the free posterior surface. On this surface a membranelike structure is present (IM). In this micrograph the structure consists of several layers. Intracytoplasmic pigment granules (PG) are present in addition to the mitochondria. PC = Posterior chamber (×6,125).

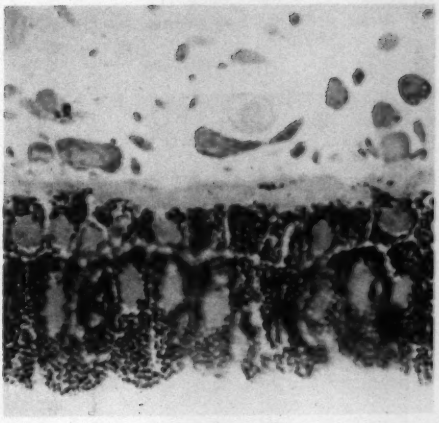
Fig. 29 (Tousimis and Fine). The posterior surface of the human iris is limited, as in the monkey, by a membranelike structure (IM). The free edge of the cells has numerous infoldings (I). Between the cells the intercellular spaces (ISp) extend and open to the surface, beneath the membrane-like structure (IM). Pigment granules (PG) almost identical in size and shape to those of the monkey pigment epithelium are present in the cytoplasm of the cells. Mitochondria (M) are present among the pigment granules and a nucleus (N) is present in the upper portion of the micrograph. PC = posterior chamber (×8,750).

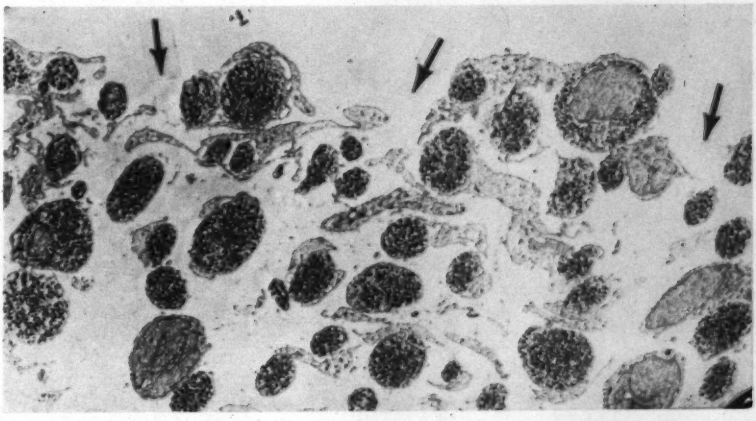




Figs. 1, 2, 3, 4 and 5 (Tousimis and Fine).
Fig. 1 (*Top, left*)
Fig. 2 (*Top, right*)
Fig. 3 (*Center, left*)
Fig. 4 (*Center, right*)
Fig. 5 (*Bottom*)







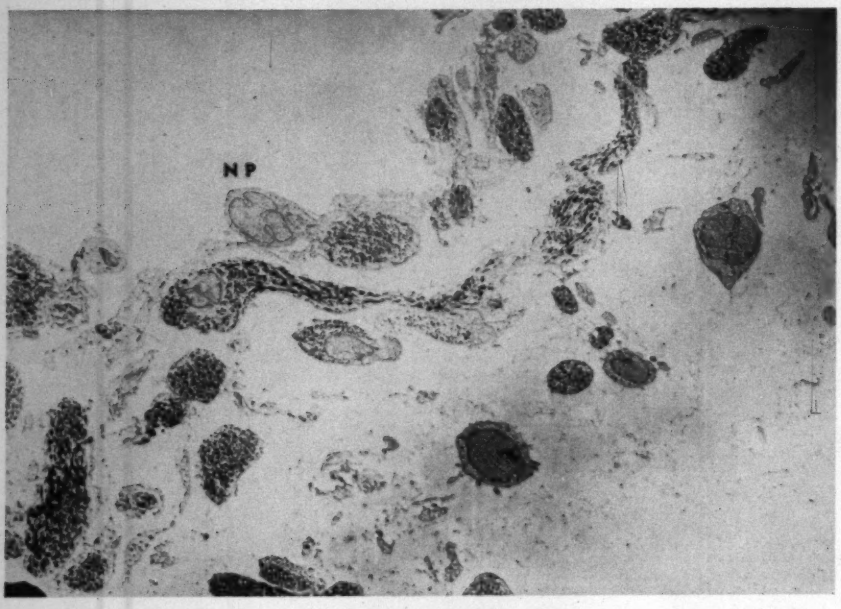
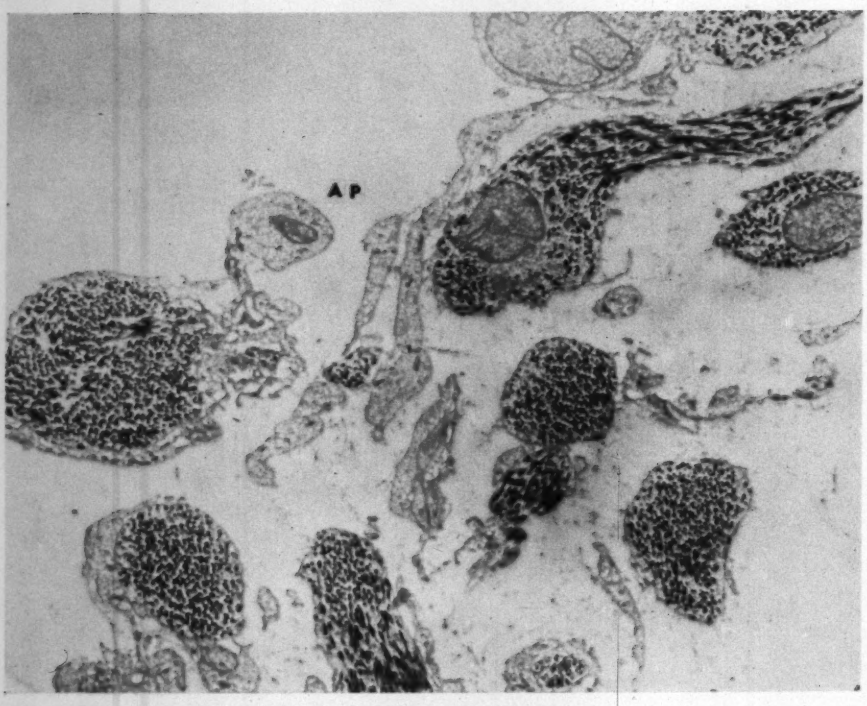


Fig. 6 (Tousimis and Fine)



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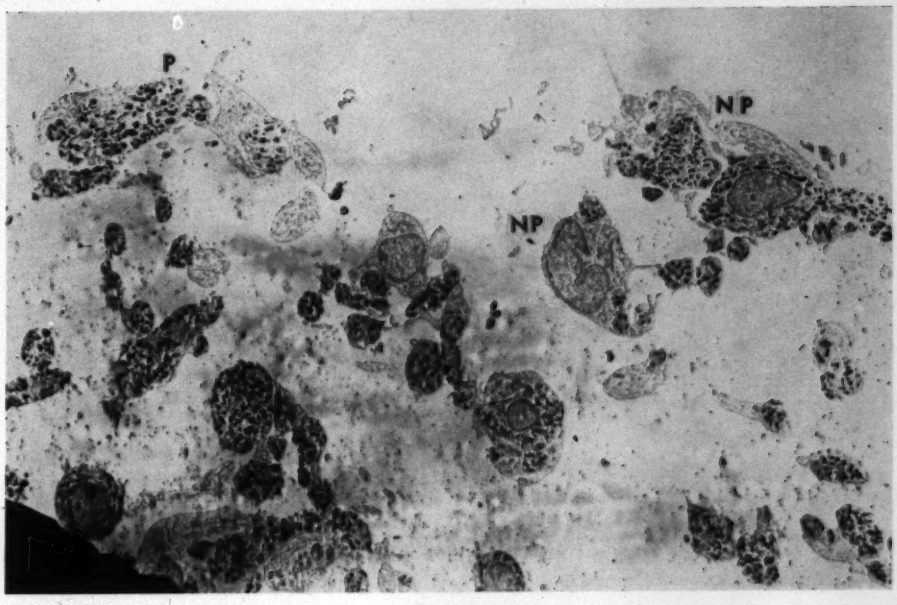

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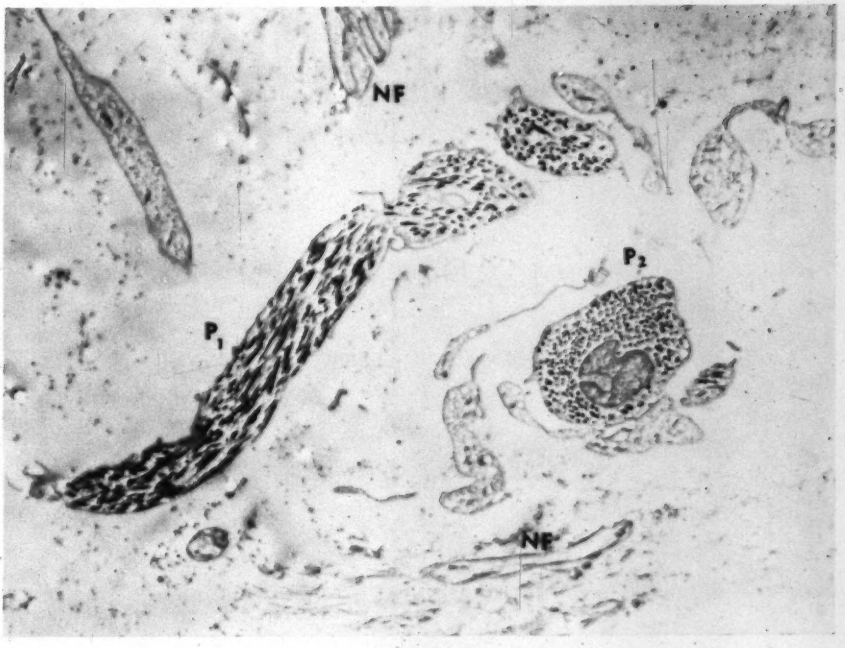

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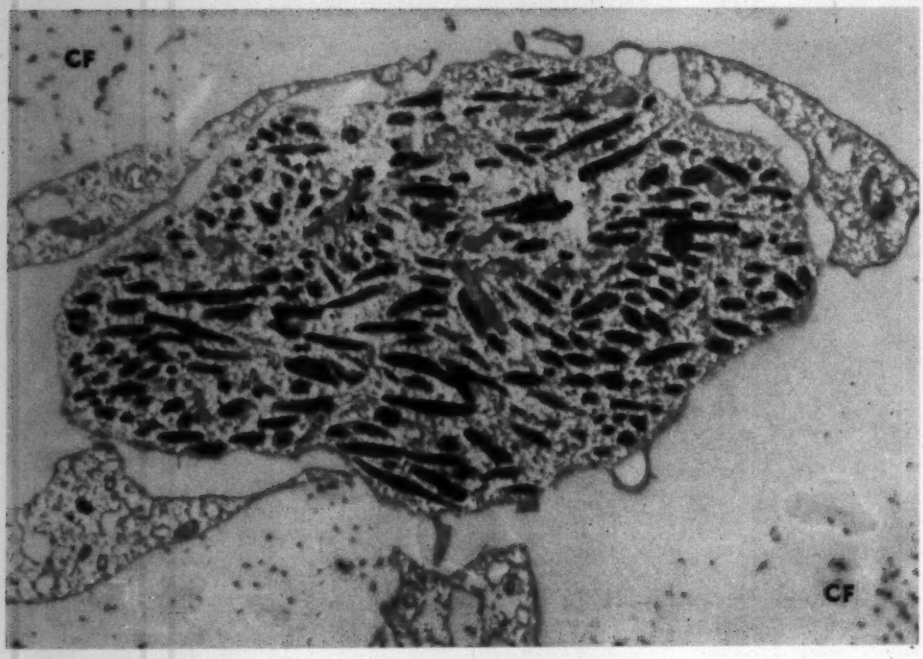


Fig. 10 (Tousimis and Fine)

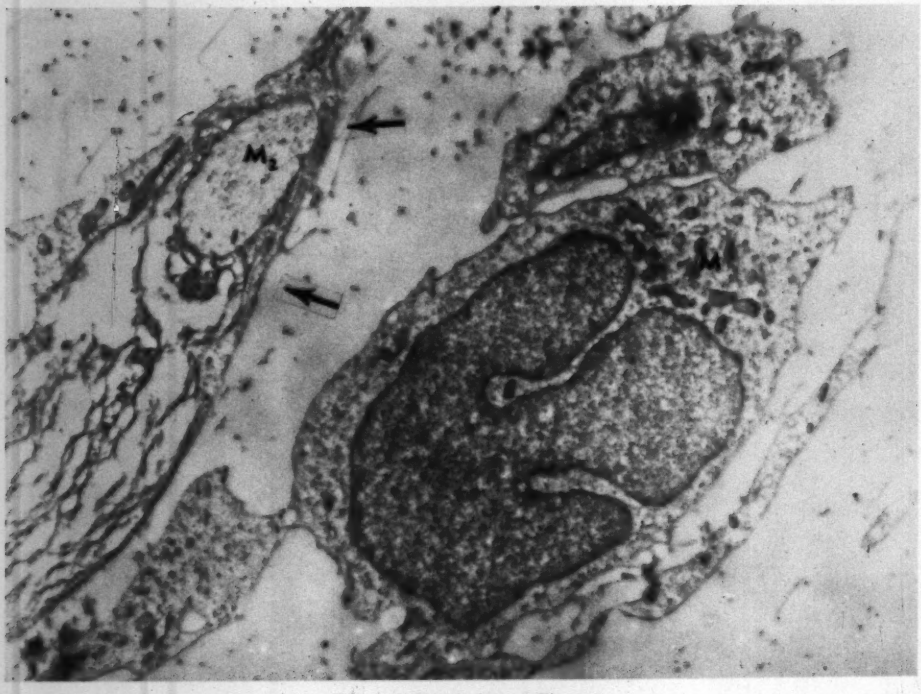


Fig. 11 (Tousimis and Fine)

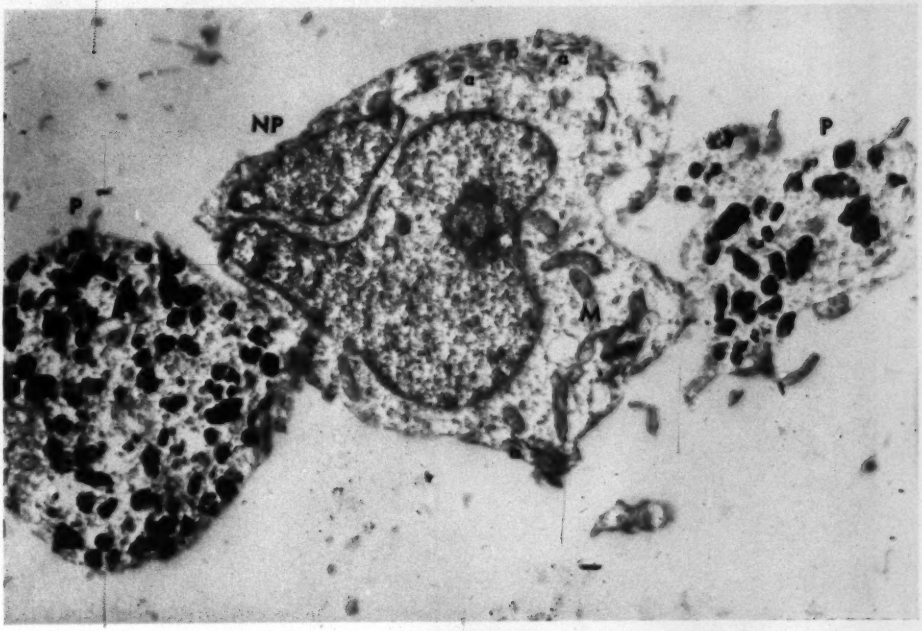


Fig. 12 (Tousimis and Fine)

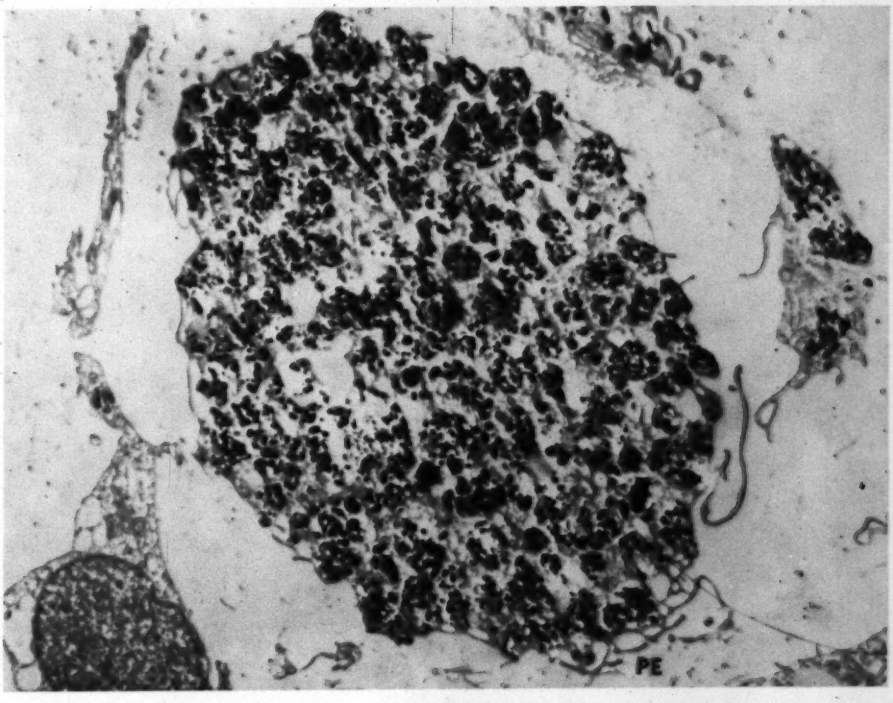
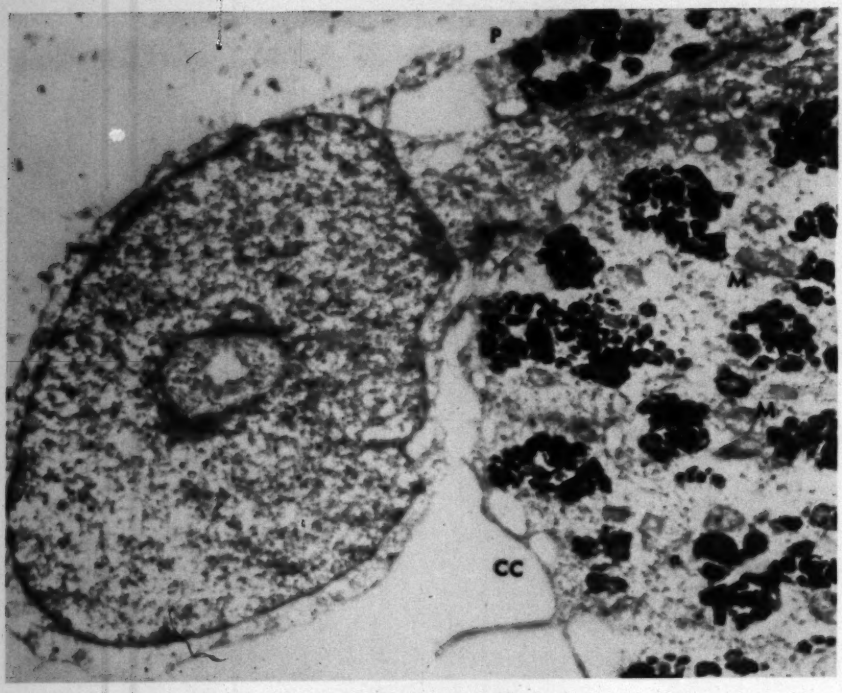


Fig. 13 (Tousimis and Fine)



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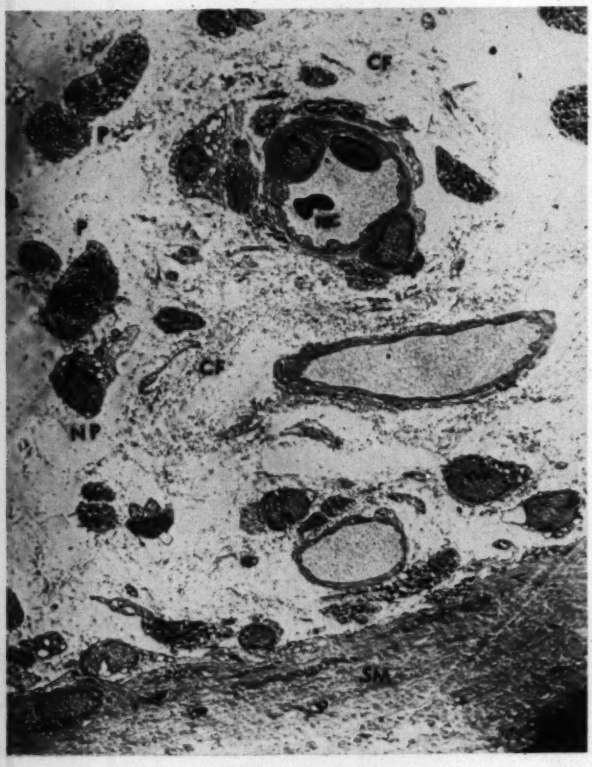
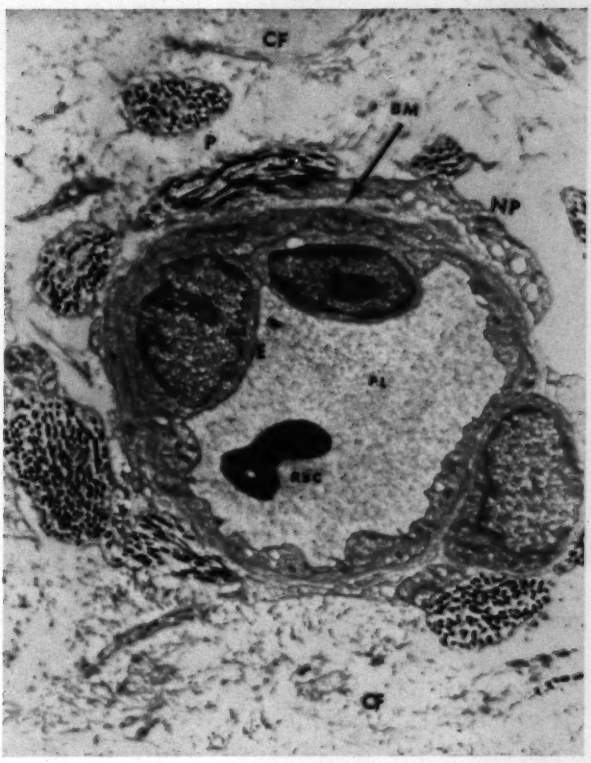


Fig. 15 (Tousimis and Fine)



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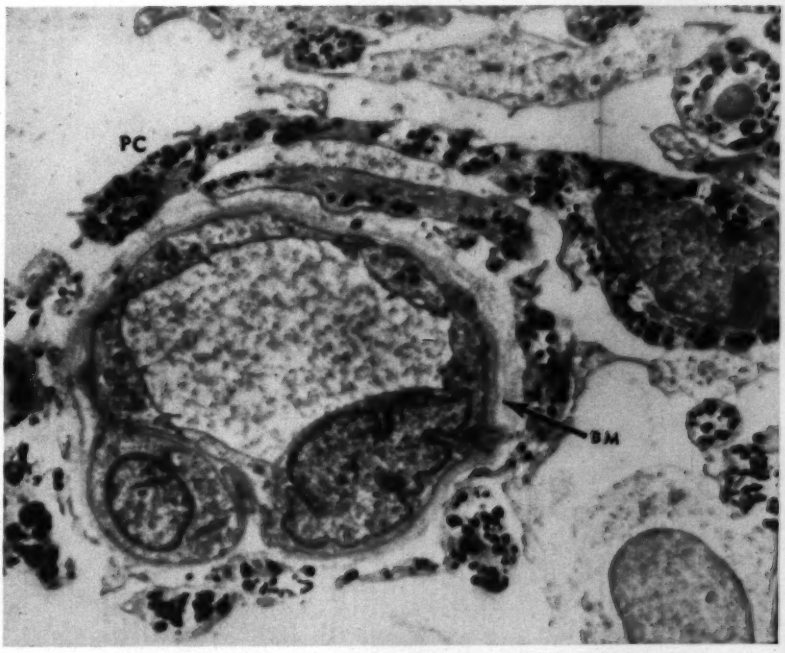

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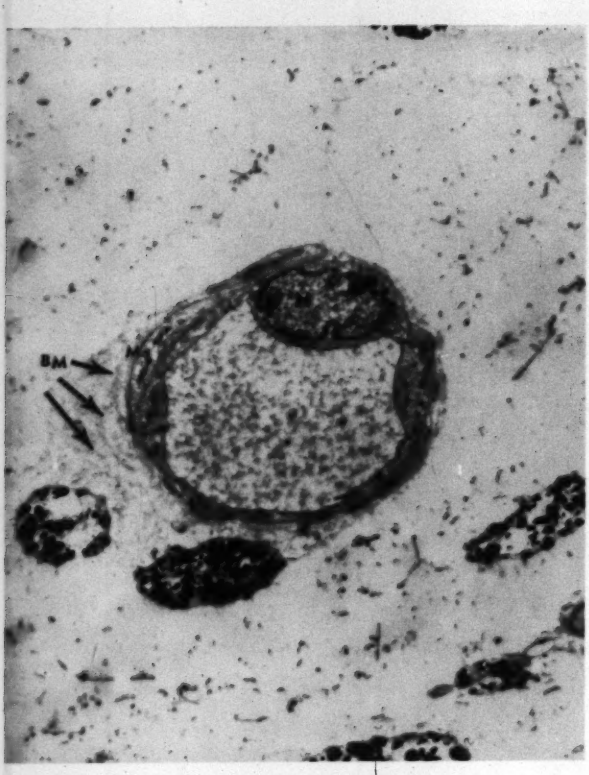

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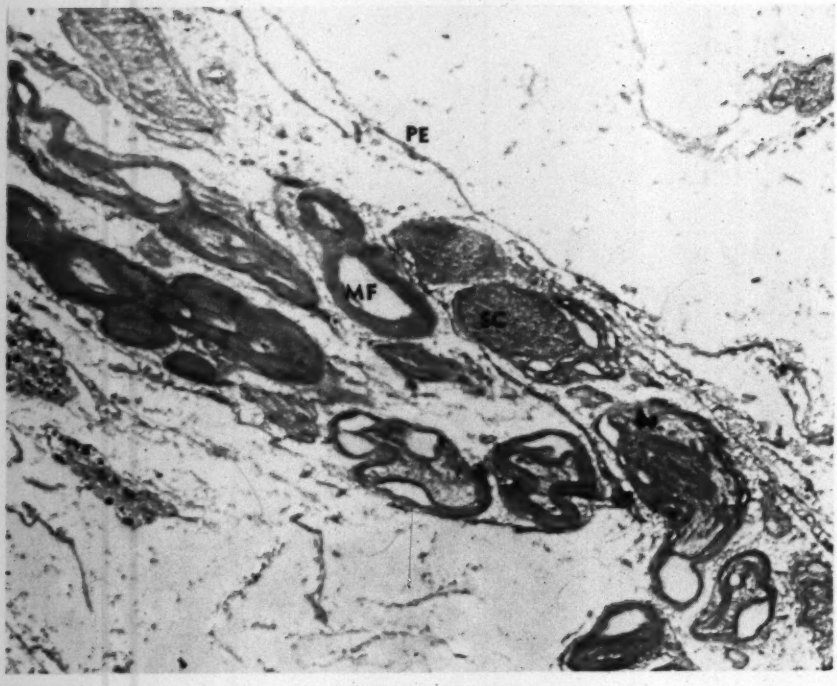

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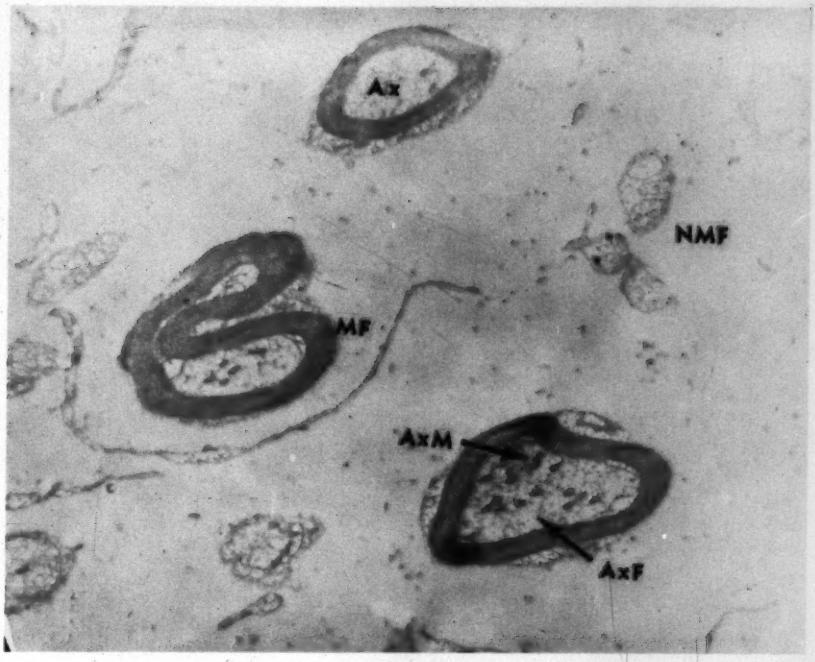

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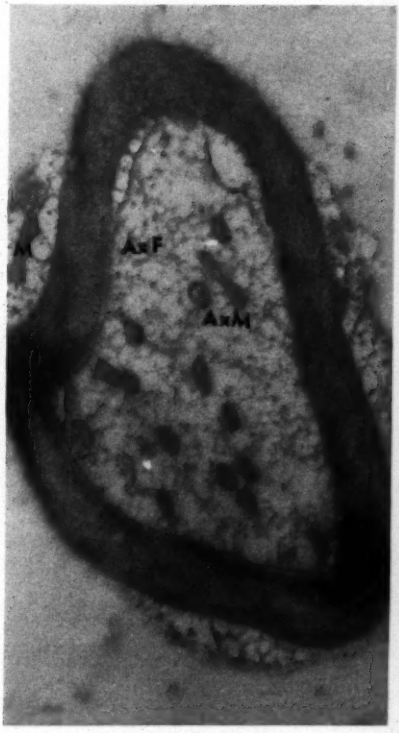

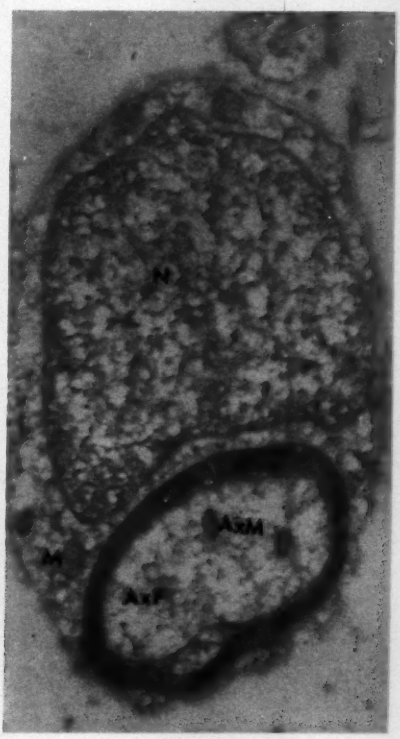

Fig. 21, *left*, and Fig. 22, *right* (Tousimis and Fine)

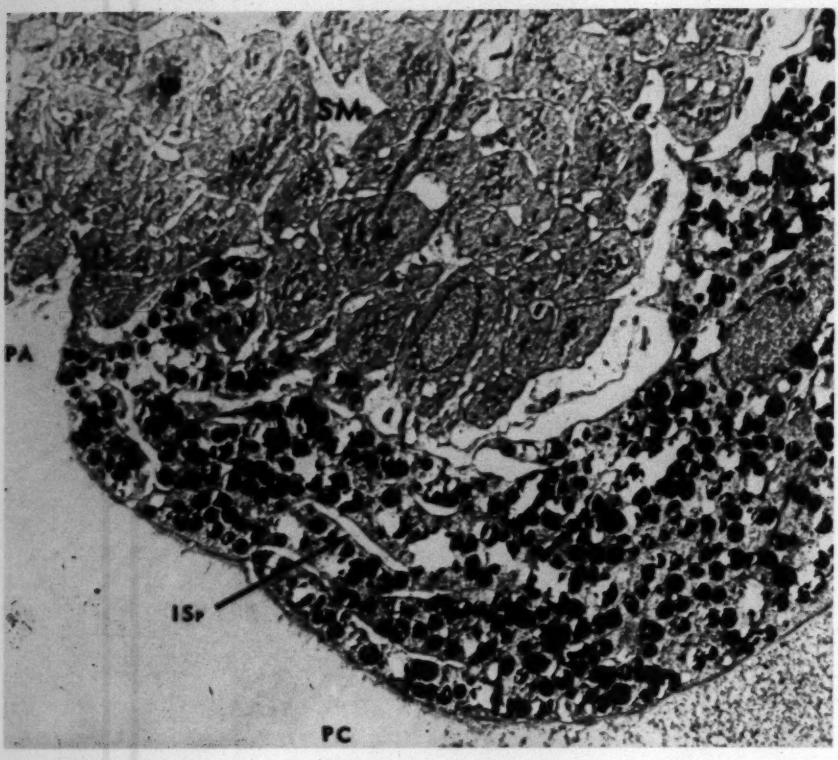

Fig. 23 (Tousimis and Fine)

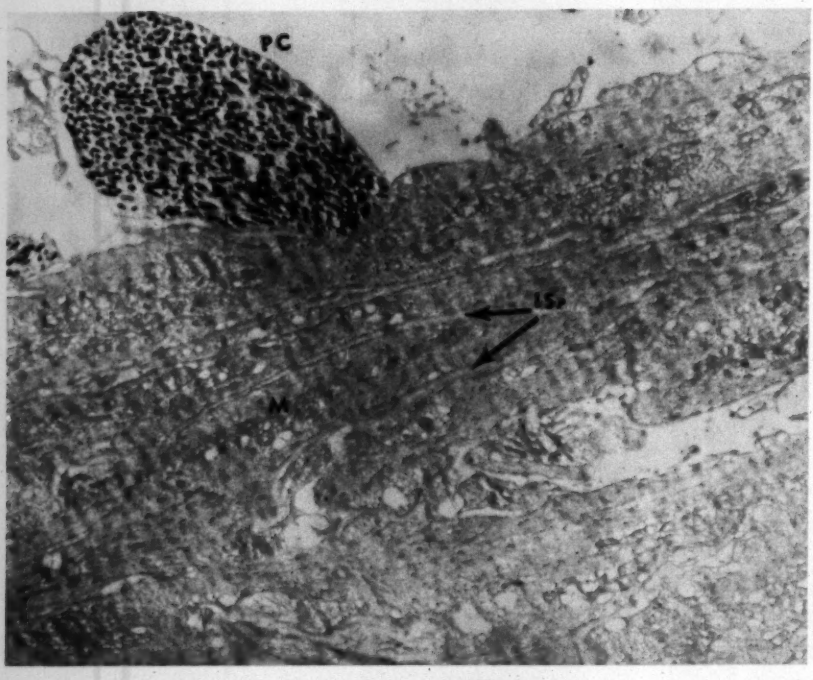

Fig. 24 (Tousimis and Fine)

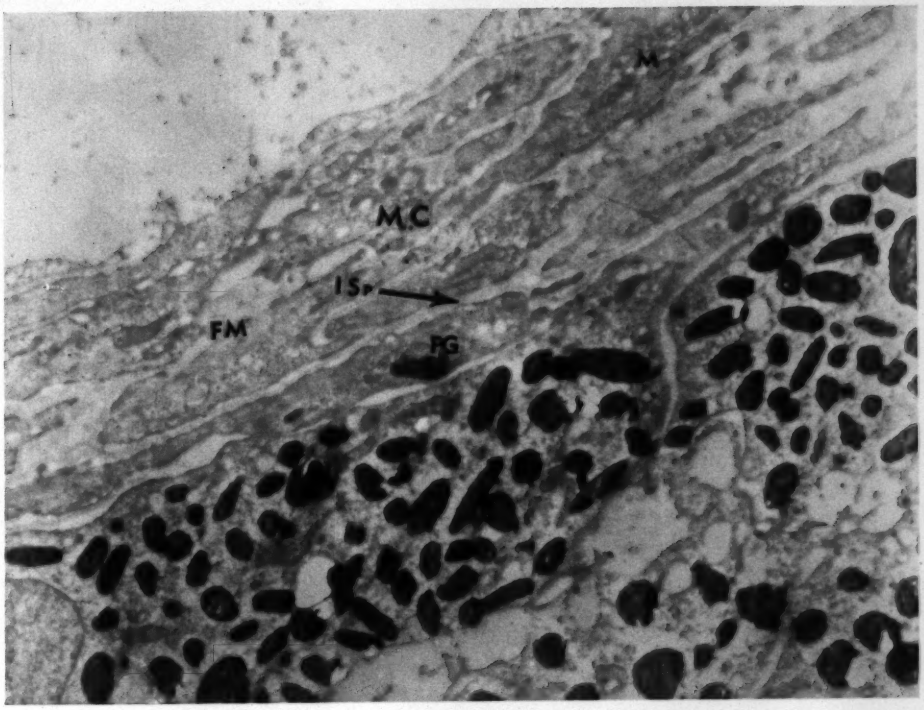

Fig. 25 (Tousimis and Fine)

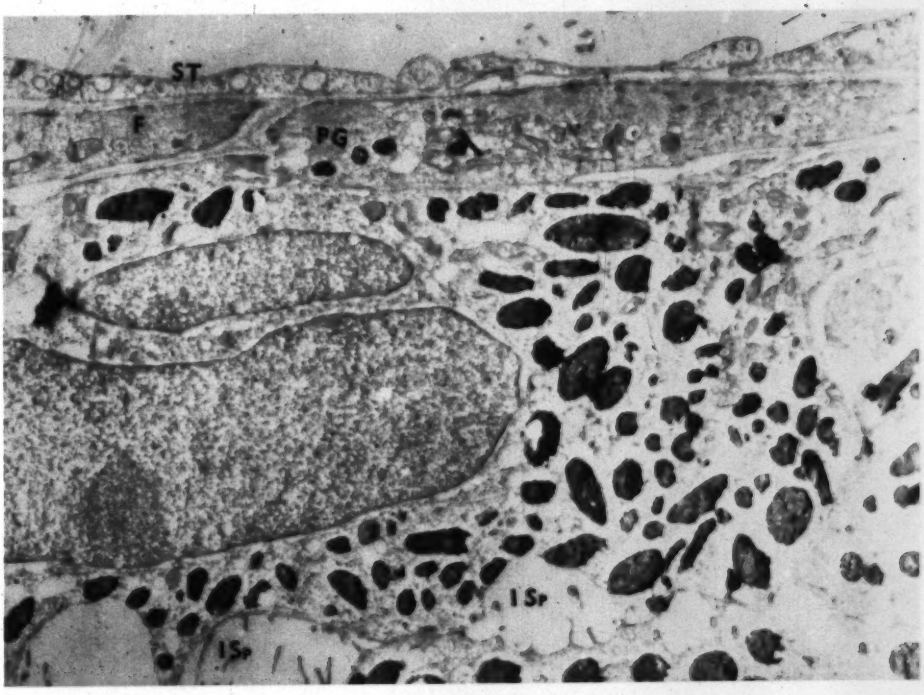

Fig. 26 (Tousimis and Fine)

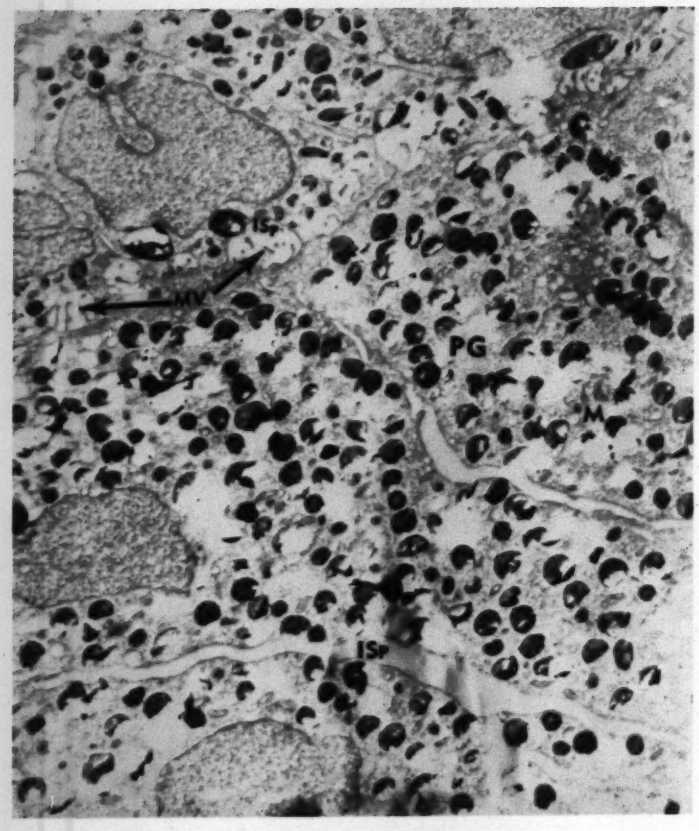

Fig. 27 (Tousimis and Fine)

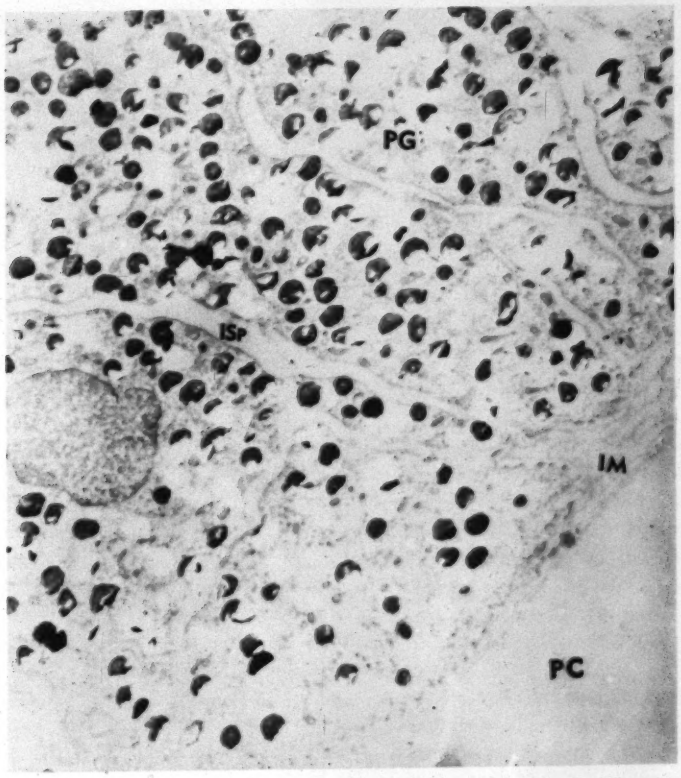

Fig. 28 (Tousimis and Fine)

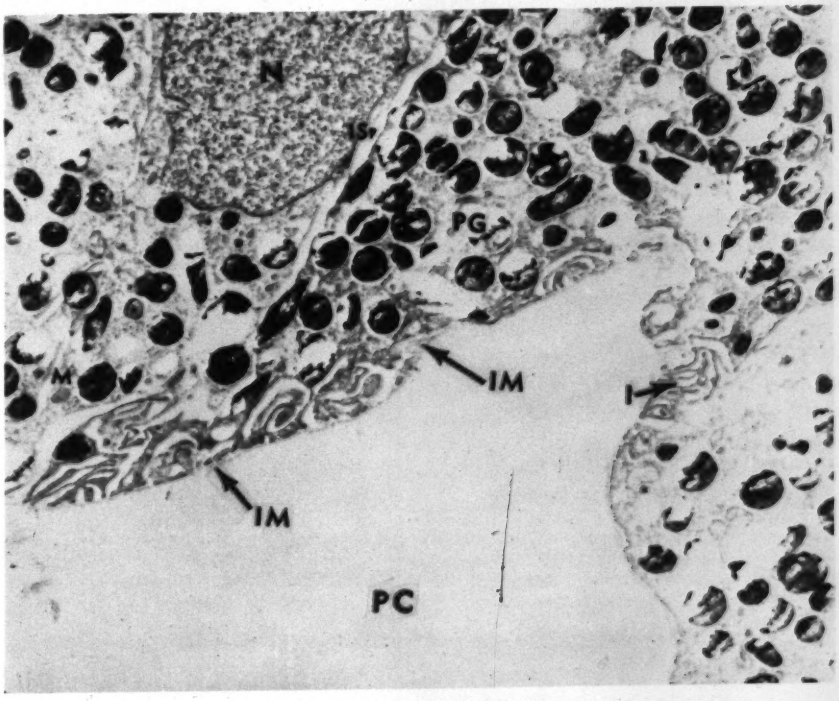

Fig. 29 (Tousimis and Fine)

THE EFFECT OF VASCULARIZATION ON THE METABOLISM*

OF THE SULFATED MUCOPOLYSACCHARIDES AND SWELLING PROPERTIES OF THE CORNEA

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The principle that tissue constituents are in a state of continuous synthesis and degradation is well established and dates from the classical work of Schönheimer.[1] Even the structural connective tissues, which are often regarded as relatively inactive, show a distinct "turnover" rate. That of the collagen is very slow, whereas the turnover rate of the ground substances is relatively rapid.

Most investigations of the metabolism of these components of the connective tissue have been made possible by the introduction of isotopic techniques. The connective tissue mucopolysaccharides may be labelled in vivo by the injection of radioactive precursors. For example, in connective tissues inorganic sulfate has been shown to be almost exclusively incorporated in compounds such as chondroitin sulfate and keratosulfate.[2]

Schiller[3] has shown that the half-life of the sulfated mucopolysaccharides in skin is approximately seven to 10 days. Dohlman[4] reported that 50 percent of the sulfated mucopolysaccharides of the sclera are replaced every 12 days, whereas those in the cornea are much more slowly "turned over," 50 percent of the total being degraded and replaced in 32 days.

It is possible that the avascularity of the cornea permits products of a degraded mucopolysaccharide to be resynthesized into a new molecule before they diffuse out of the cornea into the systemic circulation. If such is the case, one would expect a more rapid turnover of corneal mucopolysaccharides in vascularized corneas—assuming that the mucoids of such a cornea are qualitatively the same as in the avascular normal tissue.

The relation of vessels to corneal mucoids is also of interest because it has been suggested that the high concentration of mucoids may be responsible for the avascularity of this tissue.[5] The unexplained basis of the slow turnover of corneal mucopolysaccharides and their presumed relation to corneal vascularization suggests that experiments on their rate of removal in vascular corneas, their behavior in the zone of advance of ingrowing new vessels, and their rate of synthesis in corneas with well-established vessels would be of considerable interest.

METHODS

The sulfated moiety of the connective tissue mucopolysaccharides was labelled in 100 gm. rats by subcutaneous injection of S^{35}-labelled sulfate (1.0 mc./kg.). It has been shown that this substance is quickly bound in organic compounds almost exclusively as esters of kerato- and chondroitin sulfate in the cornea.[4] The location and relative concentration of the bound sulfate was determined by use of an appositional type of radioautograph of the entire cornea and adjacent sclera. Vascularization was induced by repeated instillations (one drop/five minutes for 30 minutes) of a 0.15 M alloxan solution at pH 7.0 on to the cornea of the right eye. The epithelium was first removed to insure penetration of the alloxan into the corneal stroma. The left eye was untreated and served as the control.

Langham[6] and Ashton and Cook[7] produced corneal vascularization in the rabbit by alloxan injections into the anterior chamber. The vascularization produced in these rats appears to be very similar to that which they reported in rabbits.

* From the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University. This investigation was supported by a research grant B-492 (C4) from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service.

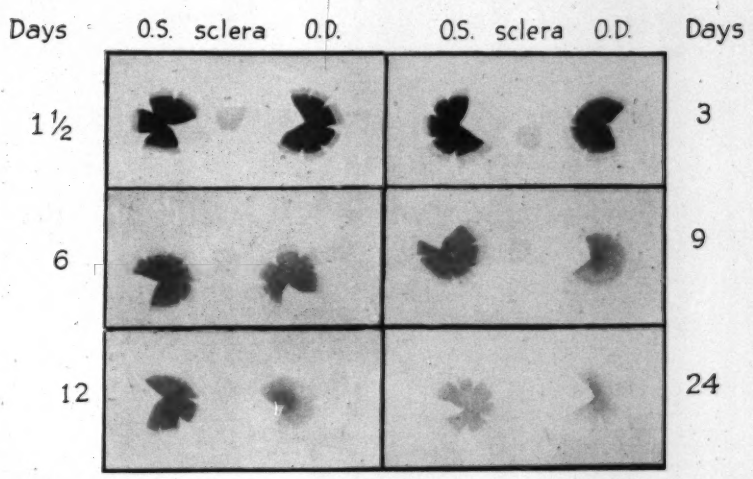

Fig. 1 (Smelser and Ozanics). Contact radioautographs of whole corneas with rim of attached sclera and a fragment of sclera from the control eye. Vascularization was produced only in the right cornea, by topical application of alloxan. The left eye was untreated. Both corneas were labelled by systemic injection of radioactive sulfate three days prior to treatment with alloxan. The animals were autopsied on the days indicated after alloxan treatment. Active invasion of the cornea by the vessels was not appreciable before the third and complete by the ninth day. Note the more rapid removal of sulfate in the vascular corneas, especially in the peripheral zone.

At autopsy the rats, under ether anesthesia, were given an intracardiac injection of 2.0 ml. of india ink which filled the corneal vessels and made them readily visible. The corneas, plus a rim of adjacent sclera and a scleral fragment from the untreated eye were dissected and radially disposed cuts made into their periphery so that they would lie flat. The tissues were washed with gentle agitation for several hours in two changes of 0.1 Molar nonradioactive sodium sulfate, and finally with distilled water in order to remove unbound isotopic sulfate.

The normal cornea and sclera, and experimental cornea, were spread flat, free from wrinkles, between two pieces of polyvinyl-

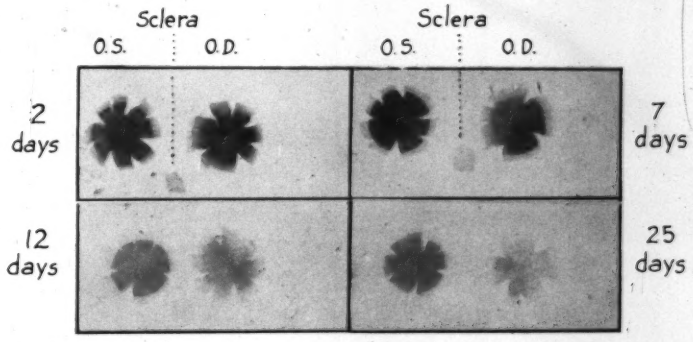

Fig. 2 (Smelser and Ozanics). Contact radioautographs of whole corneas and scleral fragment, showing the incorporation and removal of radioactive sulfate in well-vascularized corneas. Radioactive sulfate was administered systemically to rats in which the cornea of the right eye had been vascularized for 42 days. In each case the left eye was the untreated control.

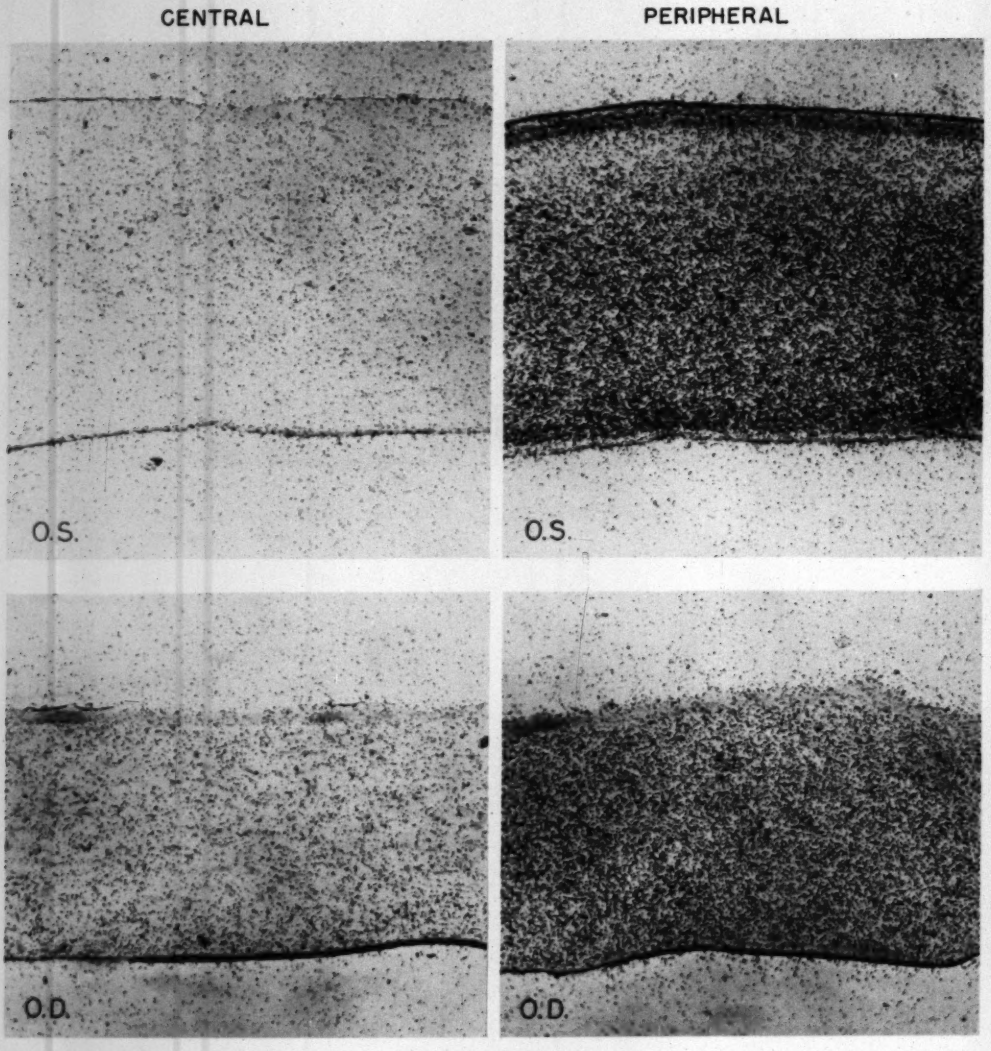

Fig. 3 (Smelser and Ozanics). Radioautographs of sections of rabbit corneas which had been labelled by the systemic injection of radioactive sulfate. The central area of each cornea had been frozen prior to the sulfate injection which resulted in negligible uptake of sulfate in this area. The peripheral portion of the cornea incorporated sulfate at a normal rate. The left eye was removed shortly after the sulfate injection, whereas the right eye was removed 30 days later. The central area of the right cornea shows that incorporation of sulfate ions had taken place during this period.

ádine film (Saran, Dow Chemical Co.). The cornea-Saran sandwich was then dried between sheets of filter paper. The thin flat preparations were placed between two autoradiographic plates (E.K.) and held firmly together under gentle pressure by rubber bands. The beta particles from the S^{35} in the tissue were allowed to act on the emulsion for one to two weeks to produce an autograph, during which time the preparations were stored in the refrigerator in light-tight boxes containing $CaCl_2$ as a desiccant.

Since the possibility exists that the type of mucopolysaccharide changes with the ingrowth of vessels, another test of the possibility that translocation of these substances occurs within the cornea seemed pertinent. It has been shown that that part of the cornea in which the cells were killed, by freezing, did not synthesize new mucopolysaccharides until it was invaded by new cells.[8] The central area (6.0 mm. in diameter) of rabbit cornea was frozen by a one minute application of a copper container filled with solid carbon dioxide-alcohol mixture (−72°C.), as reported earlier.[8] Forty-eight hours after corneal freezing the rabbit was injected intravenously with 1.0 mc. $S^{35}O_4$/kg. body weight. Four days after labelling the corneal mucoids with sulfate, one eye was enucleated and fixed in Carnoy's solution and sections prepared for the liquid emulsion coating type of radioautographs. Autographs of the remaining eye were prepared one month later to determine how the distribution of labelled sulfate had changed in the intervening time. In these experiments no vascularization of the cornea occurred.

Since the ability of corneal tissue to swell is largely dependent on its mucoid content, the imbibition of water by normal and vascular corneas was determined. The cornea of the right eye of these animals was made vascular by treatment with alloxan as described. Sixty days later the animals were autopsied, the corneas dissected free from sclera and placed in either distilled water or a Ringer-Locke solution at 0-4°C, and allowed to swell. At intervals the corneas were carefully drained of water clinging to them and weighed. Although the treated corneas were completely vascularized, the blood vessels did not add significantly to their total mass.

Results

I. Effect of vascularization on pre-existing sulfated mucopolysaccharides

The purpose of the first series of experiments was to determine the effect of vascularization on labelled material already present in the normal cornea. These rats were injected with the S^{35} sulfate and three days later the right eye of each animal was treated with alloxan as described. The animals were autopsied (five in each group) 1.5, 3, 6, 9, 12 and 24 days later.

Radioactive sulfate was always incorporated in the normal control corneas in a homogeneous manner from limbus to center and, when both eyes were normal, equally in both. The demarcation between the cornea and sclera was sharp and the uptake of sulfate by the sclera was in every case distinctly less than that by the cornea.

Study of the autoradiographs obtained in this manner (fig. 1) showed that when corneas with S^{35}-labelled mucopolysaccharides were abraded and treated with alloxan, some of the S^{35} disappeared within 36 hours. This reduction took place evenly throughout the cornea from limbus to limbus. The difference between the treated and normal cornea was slight but definite in all cases. At this stage vascularization had just started, the vessels having advanced no more than 0.1 mm. from the limbus. The corneal margin was clearly recognizable in the autographs and had not been changed by the beginning growth of the limbal vessels.

From the third to sixth day after alloxan treatment the vessels invaded the corneal stroma vigorously, but the difference between the radioautograph of the experimental and control corneas did not change significantly from that described at 36 hours. In two of the five cases studied at the six-day stage, the difference in sulfate content of the experimental and normal corneas was so slight that it was recorded as questionable. On the ninth day after alloxan treatment three of the five animals still showed only a slight reduction in the sulfate content of the experimental cornea. In two cases, however, with the same degree of vascularization, the sulfate content was somewhat more depleted. At this stage there was a vascular invasion all around the limbus reaching nearly to the center.

The amount of sulfated material found in the cornea became decidedly reduced, relative to the control, between the ninth and the 12th day, and in most of the animals the limbal zone had definitely lost more sulfate than the central area. At this stage the cornea was almost completely and evenly vascularized. On the 24th day after alloxan treatment all five of the vascularized corneas contained far less sulfate than did those of the contralateral normal control eye, and in all cases the reduction had clearly progressed more rapidly in the peripheral portion. The rate of reduction of sulfate, however, was not as rapid as that in the normal sclera.

II. Sulfate incorporation in vascularizing corneas

Although alloxan-treated vascularized corneas lose their S^{35}-labelled components at a rate exceeding normal, they also incorporate sulfate at an elevated rate. Radioactive sulfate was injected into rats in which the vascularizing process in the cornea was at its height, on the eighth day after alloxan instillation. At this time the vessels were growing rapidly but had not reached the center of the cornea. Radioautographs showed that sulfate was incorporated very vigorously in the vascular peripheral area, whereas the central avascular cornea synthesized new sulfated compounds very poorly, less well even than did the normal sclera.

Other rats in which the vascularization process was complete were found also to inporate S^{35} in the connective tissue at a rate considerably exceeding that in the contralateral normal control eye. At this stage, essentially the entire cornea was vascularized, the vessels were patent and circulation good. The cornea between the vessels appear to be transparent and the inflammatory process had subsided. In all cases the vascularized cornea incorporated sulfate at a higher rate than normal, but in four of the five animals a somewhat less dense incorporation was observed in the center.

III. Radioactive sulfate turnover in corneas with established vascularization

In this series, the right eye of the rats was treated with alloxan 42 days prior to injection of the isotopic sulfate. At that time corneal vascularization was complete and the initial inflammation had subsided. The corneas were clear and thin. Groups of five rats were killed 2, 7, 12, and 15 days (fig. 2) after injection of the sulfate to determine (1) how much sulfate had been taken up by the vascularized corneas, and (2) how quickly it was removed subsequently.

In those rats in which the cornea of one eye was vascular, a dark limbal line was seen in the autographs of the normal eye; such a line, indicating a high concentration of S^{35}, was not seen in the autographs of vascular corneas, nor was it seen in eyes which had received sulfate before alloxan had been administered.

Two days after injection of sulfate, the first group of rats was studied. In four of five of these the S^{35} uptake of the normal cornea was slightly greater than in the vascular. Sulfate incorporation was uneven in all of the vascular corneas, giving a mottled appearance to the autograph which had no obvious relation to the pattern of vessels. By the seventh day the dark limbal line of autographs of the normal corneas had disappeared. Autographs of the vascular corneas were still uneven in density or mottled, but were distinctly lighter than those of the control corneas from the same animal, indicating a more rapid loss of sulfate from the vascular corneas.

Autographs of the 12-day series showed that the scleras were almost free of S^{35}. Its distribution in the vascular cornea was still uneven, but it was obvious that sulfate loss was at a much greater rate in the vascular than in the normal cornea. There was a slight indication that this loss was more pronounced in the peripheral part.

The experiment was terminated on the

25th day, at which time the autographs of all but one sclera were negative. The vascular corneas had lost a great deal of their sulfate, far more than the normal, but, in general, not as much as the scleras. However, in two cases the rate of loss was nearly as great as in the sclera.

IV. Translocation of labelled sulfate in avascular corneas

Autographs of central and peripheral regions of corneas of one rabbit are shown in Figure 3, in which S^{35} sulfate was injected intravenously two days after the central region of both corneas had been devitalized by freezing as described. The left eye was removed two days later, and in consequence little or no sulfate was fixed in this region as shown by the autograph of the left (O.S.) cornea. Good incorporation of the sulfate was obtained, however, in the periphery of the cornea as shown in the autograph of that region. One month later the rabbit was sacrificed and the right eye (O.D.) prepared for autography. Some decrease in concentration of S^{35} material occurred in the peripheral part of the cornea and evidence of an appreciable quantity of organically bound S^{35} was found in the central zone. After freezing corneal cells migrated into the depopulated zone, and as S^{35} sulphate became available it was incorporated into corneal components in an area previously free of S^{35}. The nearest source of labelled sulfate ions was the peripheral cornea but it is possible that some of them may have been released from other tissues, for example, skin, and brought to the eye by the circulatory system. This source would be a very poor one, since active mucopolysaccharide synthesis was not active in the central zone during the first week of the experiment during which most of the sulfate from extraocular tissues was available. In addition, such ions would have a high probability of being excreted or resynthesized in other sites such as skin, cartilage and glandular mucous.

V. Swelling of vascular and avascular corneas

The ability of the vascularized cornea to swell in water was distinctly less than normal, as shown in Figures 4 and 5. Corneal swelling in Ringer-Locke solution was less than in distilled water, but the difference between the vascular and avascular cornea was clearly significant. Eight animals were used in each group. In both experiments, the distribution of the weights of the control and vascular swollen corneas differed significantly after 90 minutes swelling. The swelling curves shown are of vascular and avascular corneas from the same rats, for only one eye of each animal was treated with alloxan and was consequently vascular.

Discussion

These experiments do not support the idea that the avascularity of the cornea is maintained by its high mucopolysaccharide con-

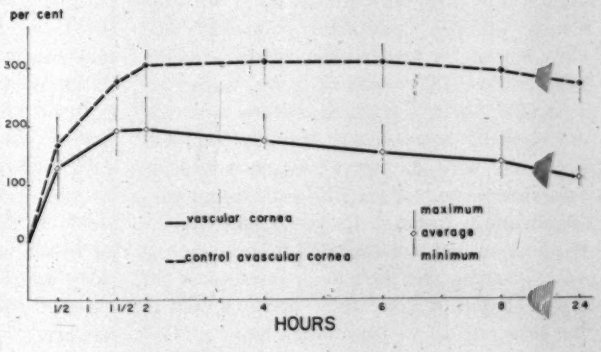

Fig. 4 (Smelser and Ozanics). Comparison of the swelling of vascular and avascular rat corneas in distilled water at 4°C. Each point represents the average of eight eyes.

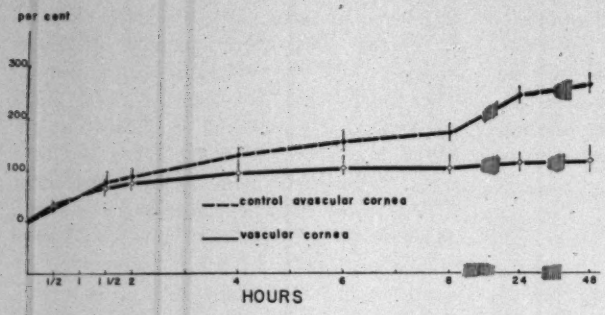

Fig. 5 (Smelser and Ozanics). Comparison of the swelling of vascular and avascular rat corneas in Ringer's solution at 4°C. Each point represents the average of eight eyes. The vertical lines indicate the range of the values.

tent which acts to prevent limbal vessels from invading the stroma. Sulfate depletion of the cornea did occur as vessels entered it, but this loss was found in an area coinciding with the vascular zone and did not take place in the zone in front of the advancing vessels. However, it must be noted that treatment with alloxan and epithelial abrasion did reduce the sulfate content throughout the entire cornea in a homogeneous manner. Possibly this reduction was enough to permit vascular invasion without a further regional reduction of sulfated mucopolysaccharides in front of the vascular sprouts. Once vessels had invaded the cornea, removal of the sulfated substances proceeded much more rapidly than in the normal tissue.

It is possible that the rapid depletion was due to the shorter distance sulfate ions had to diffuse before they entered the vascular system. However, the sulfate may have been released by destruction of the connective tissue ground substance caused by the inflammation attending the vascular invasion. Following alloxan instillation polymorphonuclear leucocytes invaded the cornea with the new vessels. There was no gross tissue destruction for the collagen stroma was not damaged by the vascular sprouts. This explanation will not serve in the series of experiments on cornea with established vascularization, because in those corneas inflammation had subsided and no new vessels were growing; yet the rate of removal of S^{35} was as rapid or even more vigorous than in the series of active vascularization.

The present experiments show that sulfate incorporation in an actively vascularizing cornea was greater than in normal. After the vascularizing process had been completed and the cornea was quiet, but contained patent vessels with circulating blood, the sulfate uptake was not higher than normal, although the rate of its turnover or metabolism was distinctly more rapid than normal. However, these data provide no indications of a change in the total amount of mucoid in the cornea. The uneven distribution of sulfate as seen in the autographs of the vascularized corneas is not explained. Such distribution could not be correlated with the vascular pattern or anything seen by clinical examination.

The rapid turnover of sulfate in vascular corneas may be due to the presence of the vessels, that is, ease of exit of sulfate ions, or to the destruction of existing corneal mucoids which may have occurred during vascularization and their replacement by a new different type with a characteristically faster turnover rate. However, experiments with the frozen cornea suggest that sulfate may move from one part of a cornea to another within 30 days.

It is difficult to believe that chondroitin sulfate or keratosulfate diffuses from the periphery to the central area, but sulfate groups once detached from such compounds no doubt do diffuse and, because the distance to vessels is so great in the normal cornea, a very appreciable proportion may be resynthesized into mucoids before they can leave the eye.

It must be borne in mind that the experiment illustrated in Figure 3 was done by systemic injection of radioactive sulfate resulting in the labelling of connective tissue throughout the body. Some of the sulfate incorporated in the previously frozen central area could have been derived from extraocular sources, as well as from the periphery of the cornea. This does not seem to be a critical objection because the experiments which suggested the long (32 days) half-life of corneal sulfate were also done on systemically labelled animals.[4] In those experiments the cornea no doubt received traces of S^{35} which had been released from extraocular connective tissues.

The basis for the slow metabolism of the sulfated compounds in the normal cornea could be attributed to an unusually low rate of metabolism of keratosulfate and, since it constitutes a large portion of the corneal mucoids, the over all turnover rate of sulfate would appear to be slow. The absence of keratosulfate from sclera and skin would, therefore, account for their difference in rate of sulfate turnover. Another possibility could be that chondroitin sulfate A is metabolized at different rates in different tissues, but to date there is no evidence in favor of this concept.

The swelling experiment shows very clearly that a fundamental change in the vascular cornea has taken place, presumably in the mucopolysaccharide fraction. The turnover studies do not indicate that less sulfated mucoids are present as suggested by the swelling experiment, but simply that they are changed more rapidly than normal.

Neither do the data of Wise[9] support the idea that vascularization is accompanied by reduction in total mucoid. In his experiments vascularization was induced by vitamin B deficient diet and the quantity of mucopolysaccharide estimated by hexosamine determinations. His data were on total content and gave no evidence of "turnover" rates.

The present data show only the amount of sulfate present, more strictly S^{35}, and changes in the nonsulfated chondroitin are not indicated; neither are possible decreases in polymerization, nor the type of sulfated mucopolysaccharide. It may be that vascularization caused the removal of either chondroitin sulfate A or keratosulfate or both followed by their replacement with another less hydrophilic sulfated material. However, this is highly speculative.

Regardless of the specific nature of the change which occurred in the sulfated mucopolysaccharides, the general structure of the vascularized cornea was not affected. After the initial edema and inflammation subsided, the corneas were transparent with details of iris structure clearly visible, although their luster appeared diminished.

Summary

1. The slow turnover of sulfated mucopolysaccharides in the cornea may be due in part to its avascularity, which permits sulfate ions, released from one combination, to be resynthesized in another molecule before they can diffuse out of the cornea.

2. Sulfated mucopolysaccharides are destroyed in a vascularizing cornea more rapidly than in the normal. Reduction in their concentration does not necessarily take place in the zone in front of the advancing vessels, but occurs slowly throughout the vascularized area.

3. Vascularizing corneas incorporated sulfate at a rate exceeding normal during the active process.

4. Sulfated compounds synthesized in a vascular cornea have a shorter life than similar material in the normal avascular structure.

5. A vascular cornea swells much less readily, in vitro, than does the avascular normal tissue.

630 West 168th Street (32).

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Discussion

Dr. Albert M. Potts (Cleveland): This report represents another in the enjoyable series of papers from Dr. Smelser's group, and sounds so very simple to do when Dr. Smelser tells about it.

Also, as a good piece of research should, it raises at least as many questions as it answers. The motive behind the work was to test two hypotheses—one, whether the cornea truly remains avascular because of its high content of mucopolysaccharide; second, whether the paradoxical slow turnover rate of mucopolysaccharide in the cornea as compared to sclera—this was work published by Dr. Dohlman and some unpublished work done independently in Dr. Smelser's laboratory—whether this difference between the two tissues, where the turnover rate in the otherwise actively metabolizing cornea is slow, and the turnover rate in the otherwise inactively metabolizing sclera is fast—could be due to the vascularization of the sclera.

The results of the experiment showed that vascularized cornea does indeed turn over or at least break down mucopolysaccharides faster than nonvascularized cornea. However, even so, the turnover in sclera was faster than vascularized cornea.

Can one compare the amount of vascularization in the alloxan-treated cornea with that in normal sclera? This quantitative dilemma still remains. True, the vascularized cornea swells less in water than the normal cornea, but the results give no direct knowledge of the nature and amount of the mucopolysaccharide in each tissue. Perhaps here, work with a larger animal and chemical determination might provide some of the needed answers.

Finally, although mucopolysaccharide is depleted in the area of advancing vessels, it is not depleted ahead of the vessels, and the question arises as to whether rarefaction is enough to allow the vessels to advance, or whether the mucopolysaccharide theory is untenable. This does not deal at all with that interesting little ring that appears at the limbus of the control eye when the experimental eye is operated on.

In each case this work opens vistas of new experiments to be done, and one hopes that they will be the subject of future reports by Dr. Smelser's group.

SOME CHARACTERISTIC COMPONENTS OF THE CILIARY EPITHELIUM*

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Electron microscopy has revealed characteristic structures in the ciliary epithelium (Holmberg, 1957; Pappas and Smelser, 1958). This short communication is con-

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† Fight-for-Sight Fellow of the National Council to Combat Blindness.

fined to only two constituents, the β-cytomembranes and the small cytoplasmic vesicles.

The β-cytomembranes are present within two regions of the epithelium, the most basal area of the pigment epithelium and the most apical part of the non-pigmented epithelium. They appear on sections as narrow folds of that part of the cell membrane which faces the external and internal limiting membrane respectively. They are present in adult eyes of all species examined, including rabbit, monkey and man. However, in rabbits one and five days of age, β-cytomembranes are rarely found and those present are very short ones. By 30 days of age the rabbits have a normal number of such membranes.

The β-cytomembranes are characteristic but not unique for the ciliary epithelium. They are also found in the renal tubules (Rhodin, 1954), the choroid plexus (Pease, 1956) the thyroid gland (Ekholm and Sjøstrand, 1957) and the pigment epithelium of the retina (Garron, unpublished). Thus, this particular cell component is found in cells where transport of water and ions takes place through the cells. It is therefore believed to be of importance for this transport. Of interest is that in the ciliary epithelium, β-cytomembranes are present at those places where fluid enters and leaves the epithelial cells.

Small vesicles (about 1000 Å in diameter) are found in the cytoplasm of both epithelial layers. They are scattered all over the cells and are often lined up in rows, particularly close to the surface of the nonpigmented epithelium. Vesicles are present in rabbits from birth and it is interesting to note that in animals, where β-cytomembranes are almost absent, the vesicles sometimes are arranged in rows as in adult animals.

After partial inhibition of secretion of aqueous humor in rabbits by Diamox, there is an enormous increase in the number of vesicles. However, this remarkable change is observed only in the nonpigmented epithelium. The pigment epithelium remains unchanged. The accumulation of vesicles is most pronounced 15 to 30 minutes after intravenous administration of Diamox. After longer periods the number of vesicles decreases to about normal. In human material the same increase in number of vesicles is noted after systemic administration of Diamox but limited exclusively to the pigment epithelium. The human nonpigmented epithelium is not altered.

The regular arrangement of the vesicles in rows near the surface of the nonpigmented epithelium is very striking and suggestive of a close relationship between the vesicles and the β-cytomembranes. However, from a static picture it is not possible to decide the nature of the relationship. Pappas and Smelser (1958) have interpreted the appearance of the vesicles in rows after Diamox administration as a sign of a breakdown of the membranes. There is also the possibility that the membranes can be formed by fusion of the vesicles. However, the number of β-cytomembranes in the Diamox-treated material is roughly the same as in the control material. This suggests that the membranes are not broken down.

The accumulation of vesicles after suppression of the secretion of aqueous humor by Diamox is correlated to the decrease in intraocular tension. This may be interpreted as the vesicles carrying aqueous, which may be formed within the cells before it reaches the posterior chamber.

The difference in effect of Diamox on rabbit and human ciliary epithelium is interesting and may be related to the differences in composition of the aqueous humor in the two species. However, so far only one human eye has been examined and more material must be studied before any definite conclusions can be drawn.

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Discussion

Dr. George D. Pappas: Dr. Holmberg's study shows very well that we are able to visualize certain aspects of physiologic phenomena with the electron microscope. In these very good electron micrographs Dr. Holmberg has demonstrated that the fine structure of the ciliary epithelium is changed or altered when secretion of aqueous is partially inhibited after Diamox.

The principal change that Dr. Holmberg has reported is the large increase in the number of vesicles in the cytoplasm of the cells facing the posterior chamber after Diamox treatment. Dr. Holmberg suggests that the vesicles are the carriers of aqueous and that during partial inhibition of secretion vesicles appear in the cytoplasm.

Dr. Smelser and I have been engaged in similar studies which we reported at this meeting last year and, by and large, our results are similar (Pappas, G. D., and Smelser, G. K.; Am. J. Ophth., **46**:299, Nov. Pt. II, 1958). By its very nature, electron microscopy can deal only with fixed, nonliving material. Therefore, one must interpret dynamic processes from static pictures. It is on this aspect of Dr. Holmberg's remarks that I would like to comment.

We have altered aqueous secretion with Diamox, as well as by other more drastic methods. In all these experiments the cell lost its characteristic infoldings and interdigitations, and vesicles appeared at first in linear arrangement in the same areas. It appears to us that the most reasonable way to explain the linear arrangement of vesicles is that they arise from the fragmentation of membranes. These changes appear to be less pronounced in Diamox treatment, but when compared with the other experiments they appear to be similar.

I believe one must use caution in stating in which direction the vesicles in the ciliary epithelial cells are moving, or if they are moving at all.

In other cells when we know the direction of fluid movement, such as the contractile vacuole in freshwater amebas, the vesicles which surround and empty their contents into the contractile vacuole are not arranged in linear fashion (Pappas, G. D., and Brandt, P. W., J. Biophys. Biochem. Cytol., **4**: 485, 1958).

Dr. V. Everett Kinsey (Detroit): I would like to point out an interesting correlation between some of the chemical findings we have made and Dr. Holmberg's observation of the increase in vesicles in the ciliary epithelium of the rabbit eye.

Dr. Reddy and I have been studying the rate of turnover of total carbon dioxide in the aqueous humor of rabbits and have found it to be extremely rapid, as did Dr. Green who reported his observations last year before this Association in San Francisco.

Following Diamox the turnover rate of total carbon dioxide decreases significantly in both the posterior and anterior chambers. We were interested in determining the time course of the inhibiting action of Diamox and, interestingly enough, found that the maximum effect of Diamox occurred between 10 and 15 minutes after intravenous administration. To our surprise the effect is all over and done with after about one hour.

Dr. Becker and his associates kindly offered to repeat some of their experiments and determine again the time course of the pressure-lowering effect of Diamox, and also the time course of the reduction in flow. The time course of these effects were distinctly different from that of the turnover of total carbon dioxide. They found that the flow rate and the intraocular pressure remains low for at least two hours after the animals were given Diamox.

Now Dr. Holmberg finds that the maximum increase in the number of vesicles in the ciliary epithelium occurs about 10 or 15 minutes after giving Diamox and the increase in vesicles disappear after about one hour. Both of these observations correlate well with the time course of the inhibitory effect of Diamox on the turnover of total carbon dioxide. I think this is extremely interesting.

If I may speculate for a moment on the point Dr. Holmberg just touched on—and I would remind this audience that in the human being the bicarbonate is deficient in the aqueous and the chloride is in excess,

whereas, in the rabbit the bicarbonate is in excess and the chloride is deficient.

Dr. Holmberg mentioned briefly, in passing, that the increase in vesicles occurred in the nonpigmented cells of the epithelium in the rabbit and in the pigmented cells in the human being. This correlation with the different distribution of bicarbonate seems to me to suggest that accumulation of vesicles has to do more with the bicarbonate formation than flow and depression of the pressure.

As Dr. Smelser mentioned earlier today the excellent electron microscopic pictures with the high resolution solve some problems but create more new and very interesting ones.

OCULAR TUMORS PRODUCED IN MICE BY METHYLCHOLANTHRENE*

II. The effect of reoperation and diathermy on tumor production

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Introduction

Methylcholanthrene, a potent chemical carcinogen, has been previously introduced into the eyes of pure strain mice.[1] A variety of intraocular and extraocular malignant tumors were observed. These have consisted of squamous cell carcinomas of the cornea, conjunctiva, and of epithelial downgrowths, malignant tumors of the iris and choroid thought to be melanomas, sarcomas of the orbit and adenocarcinomas of the harderian gland.

The present experiments were designed to evaluate further the effects of the implantation of methylcholanthrene (MCA) impregnated silk suture in the posterior segment of the globe. The possible tumor promoting action of either a repeat operation with implantation of another MCA impregnated suture or of a diathermy puncture after original operation has been tested. Under the conditions of these experiments a repeat operation with either of these procedures did not alter significantly the tumor yield obtained by a single operation.

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Subjects and Methods

C_3H (Cumberland Farms) mice ranging from two to five months of age were used exclusively. Methylcholanthrene was impregnated in 4-0 black silk atraumatic eye sutures as previously described.[1] The needle was introduced through the sclera at about the equator. The suture was threaded through the posterior segment between the lens and retina and out the opposite side of the globe. The ends of the suture were cut at the scleral surface and the free ends allowed to retract into the globe. One hundred eyes of 50 animals were operated; 52 eyes were reoperated with the identical technique 21, 28 and 36 days later; 48 eyes received a penetrating diathermy puncture at the approximate site of the suture 16, 23 and 30 days after the insertion of the methylcholanthrene suture. A standard Walker retinal detachment unit (Rose Electrical Products) served as a current source. A 1.0 mm. penetrating electrode was applied to the sclera with 30 ma current for two seconds. Sixty-four C_3H eyes were operated by a single implantation of MCA suture to serve as controls.

Results

The data collected in these experiments are tabulated in Tables 1 and 2. The difference in total tumor yield of 8.5 percent ob-

TABLE 1

MCA SUTURE IMPLANT AND SECOND OPERATION

Number of Eyes Operated	Second Procedure		Days Between 1st & 2nd Procedure	No. of Tumors	% Tumor Yield
	a. Re-operation with Suture	b. Diathermy			
16	—	16	16	0	0
8	8	—	21	1	12.5
12	—	12	23	1	8.5
20	20	—	28	0	0
20	—	20	30	3	15
24	24	—	36	5	20.8

tained by initial operation followed by diathermy, with 11.5 percent for reoperation with a second MCA suture is not statistically significant. The controls that were operated with a single MCA suture developed tumors in 9.3 percent of operated eyes. Table 1 shows that the tumor yield in general increased as the period between initial MCA suture implantation and the secondary procedure was increased. The figures are as yet inadequate to draw definite conclusions on this point. Further experiments are now in progress. Approximately one third of the eyes that were operated by implantation of a single MCA suture ultimately became phthisical within two months of operation and were lost. In those eyes receiving either a second suture implantation or diathermy, the number of eyes ultimately lost from phthisis bulbi was approximately 50 percent.

Discussion

Before discussing the data collected here, some observations that are fundamental to the problem of co-carcinogenesis and carcinogen promoters are cited. Cramer and Simpson, 1945,[2] noted that following the skin application of a single *small* dose of carcinogen, inadequate to produce tumor, that the initial effect of local irritation occurred but following the usual latent period, no tumors developed. However, if following the single application of this same small amount of carcinogen the non-specific effect of burning the skin was added, tumors developed at the burn site. Deelman, 1924,[3] showed that tumors occurred at the edge of a deep skin wound inflicted following the application of a *small* dose of carcinogen that was normally insufficient to produce tumors. Yet repeated application of injury or non-specific irritants alone do not produce tumors.

These observations together with their own similar data led Berenblum and Shubik[4] to conclude that the initial irritant action or "initiating action" is a specific one and is only produced by a definite carcino-

TABLE 2

TABLE OF INDUCED TUMORS

Procedure	No. of Eyes Operated	No. of Tumors	Percent Tumor Yield	Classification of Tumor		
				Squamous Cell Carcinomas of Conjunctiva	Choroidal Tumors	Adenocarcinoma of Harderian Gland
Single implantation of MCA suture	64	6	9.3	2	3	1
Repeat implantation of MCA suture	52	6	11.5	6	0	0
MCA suture plus diathermy	48	4	8.5	4	0	0

gen. Once the "initiating action" produced by a specific carcinogen has occurred in the tissue, then a nonspecific irritant, for example, heat or trauma, may exert a "promoting action" and neoplasia follows. They reasoned that the specific carcinogenic chemical changes a few normal cells into latent tumor cells (initiating action) on a single application. If the initial irritant effect is potent enough, tumors will develop without a secondary promoter. If the initial carcinogen is applied in a very small amount, only the initiating effect occurs. Either a repetition of the carcinogen or the application of a nonspecific irritant or "promoter" will then be required to induce neoplasia. These so-called promoters are usually designated co-carcinogens.

The co-carcinogens, though not themselves necessarily carcinogenic, may also act during the initial application of the carcinogen.[4] Their effect can be (1) to shorten the latent period of actual neoplasia where the initial carcinogen is sufficiently potent to produce tumors and (2) promote the production of tumor where the carcinogen dosage itself is insufficient.

The average tumor yield of 10 percent obtained where a secondary procedure followed the original MCA implantation is almost the same incidence (9.3 percent) as produced by a single procedure of MCA implantation. The second implantation of an MCA suture or of diathermy application following the initial procedure was ineffective here as a promoter or co-carcinogen. It is possible, however, that the larger number of phthisical eyes developing in the multiple procedure groups may explain in part the failure to increase the final tumor yield in these experiments.

It is significant that where the eyes were re-operated by either diathermy or a repeat implantation of a MCA suture that tumor formation occurred exclusively in the overlying conjunctival epithelium with squamous cell carcinomas developing. Possibly these epithelial tumors may have developed early and destroyed any latent or slower growing intraocular tumors. Examples of the differ-

Fig. 2 (Patz, et al.). Right eye of operated animal on right is proptosed and orbit is filled with tumor. Operation consisted of single implantation of MCA suture. Sections showed squamous cell carcinoma originating from conjunctiva. Unoperated control animal is on left.

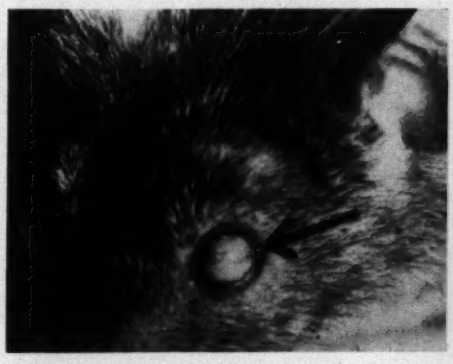

Fig. 1 (Patz, et al.). Tumor mass fills entire globe of operated left eye. This tumor later ruptured globe and filled orbit. Sections showed malignant tumor apparently of choroidal origin and resembling melanoma.

Fig. 3 (Patz, et al.). Subcutaneous transplant from tumor seen in Figure 2. Original piece of transplanted tumor measured 1 mm. in diameter. Growth after two months measures 16 by 22 by 10 mm.

Fig. 4 (Patz, et al.). Medium-power view of section of one of choroidal tumors having characteristic spindle cell arrangement. Note similarity of this animal tumor to spindle B human melanoma. (×100, hematoxylin-eosin.)

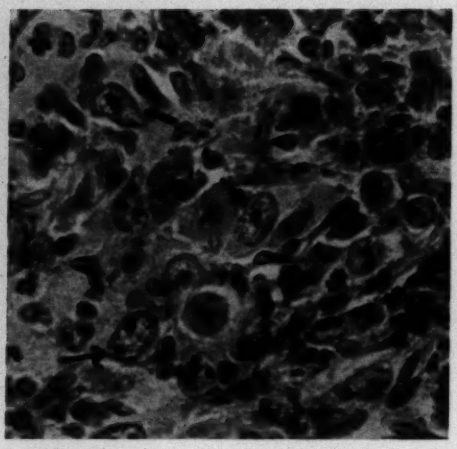

Fig. 5 (Patz, et al.). High-power view of section of one of choroidal tumors. Arrows point to epithelioidlike tumor cells resembling epithelioid human malignant melanoma. (×500, hematoxylin-eosin.)

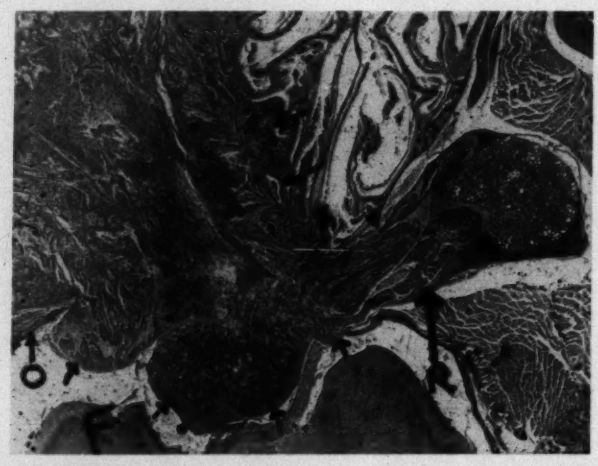

Fig. 6 (Patz, et al.). Intracranial extension of a squamous cell carcinoma of conjunctiva. Tumor fills orbit and erodes through orbital wall (O) to displace frontal lobe (F). Tumor extends across base of skull to invade R orbit (R). (×60, hematoxylin-eosin.)

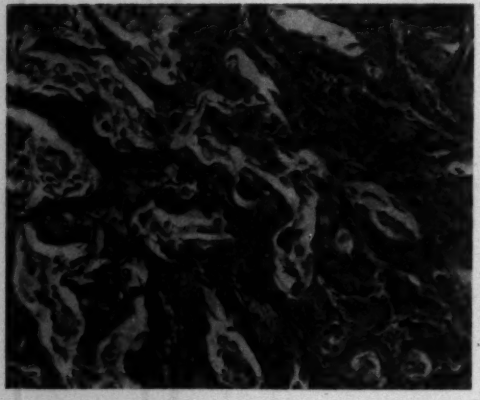

Fig. 7 (Patz, et al.). Higher power view of tumor seen in Figure 6, showing cellular details of tumor. (×400, hematoxylin-eosin.)

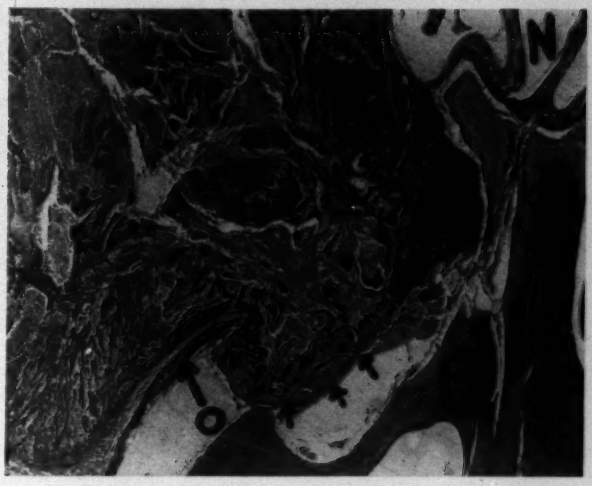

Fig. 8 (Patz, et al.). Intracranial extension of a squamous cell carcinoma of conjunctiva. (O) orbital wall. Note atrophy and destruction of fore brain (B). (×60, hematoxylin-eosin.)

ent types of induced tumors are seen in Figures 1 through 9.

No distal metastases have as yet been recorded from the primary ocular tumors. Invasion of the intracranial cavity occurred in several tumors and the animals died shortly thereafter. In one instance (fig. 6) the tumor spread across the base of the skull intracranially to reach the opposite orbit.

Summary and Conclusions

1. Methylcholanthrene, a carcinogenic hydrocarbon, has been introduced into the eyes of several strains of mice and a variety of intraocular and extraocular tumors have been produced.

2. Repeated transplantation of several of the tumors to other mice of the same strain was successful. The local malignancy of the tumors was evident by their rupturing the globe and invading the orbit and in some instances by intracranial extension of the tumor. No distal metastases have been thus far observed.

3. A repeated introduction of a methyl-

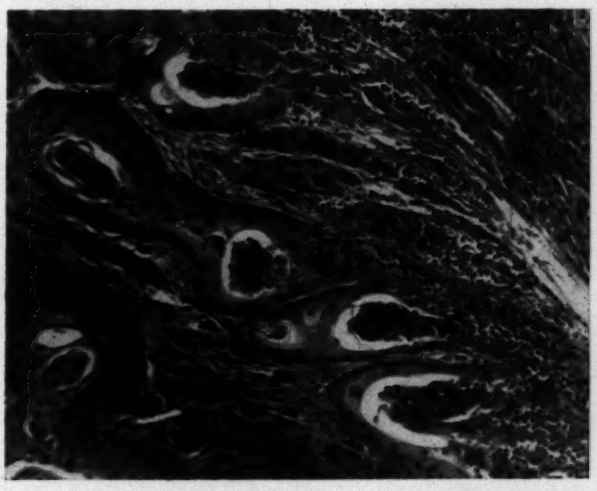

Fig. 9 (Patz, et al.). Higher power view of tumor seen in Figure 8 showing cellular pattern of tumor. (×400 hematoxylin-eosin.)

cholanthrene impregnated suture after initial operation failed to increase the tumor yield. Penetrating diathermy applied after initial introduction of a methylcholanthrene suture was also ineffectual. It was concluded that the re-operation or diathermy as utilized in these experiments failed to act as a promoter or co-carinogen on the original MCA suture operation.

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Discussion

Irving H. Leopold, (Philadelphia): Dr. Patz and his co-workers have been able to induce the growth of tumors in ocular tissues. The yield has been small but definite. His attempts to increase the initial yield by additional methods of carcinogenetic stimulation have not succeeded to date. However, there are many other enhancement methods that can be employed which he will no doubt try.

In the present paper, the author does not indicate the method of impregnation of silk suture. It would be helpful perhaps to know the kind of solution which was employed; the solvent and the percent of it; whether the suture was impregnated with powder of ethylcholanthrene. It might be rewarding to know whether the variations in techniques of impregnation and introduction made a difference in the incidence of tumor production.

Dr. Kaczurowski, working at the Wills Eye Hospital, has been using 3-methylcholanthrene in an attempt to induce ocular tumors as well as tumors in other areas of the body. He has placed pellets into the brain or external ear of the mouse as well as into the ocular tissue. Rabbits have also been employed in his studies. These pellets have remained in the injected area from 6 months to a year. This method has rarely produced any ocular tumors and it is interesting that the suture method in which the contained carcinogenic solution could be lost in a few days is so effective. Could it be that the presence of the silk sutures act as a foreign body in the eye and that this could be interpreted as a co-carcinogenic agent perhaps by virtue of its mechanical irritation? Perhaps the success could vary with the strain or specie of animal employed.

Reoperation and diathermy in comparison with the size of the mouse eye are relatively massive and strong insults. Perhaps the reaction of the secondary interference causes such great metabolic and physiologic changes in the eye that they prevent any increase of tumor stimulant in comparison with the control group. It would be interesting to know whether or not the diathermy and reoperation causes strong inflammation and exudation.

The importance of this work is considerable. It demonstrates that methods employed in other body tissues will also work in ocular tissues. Perhaps this indicates that these ocular tissues might respond, as other body tissues, to therapeutic inhibitors or suppressors of carcinogenesis.

It is difficult to evaluate chemotherapeutic and surgical methods in the control of intraocular and extraocular malignancies. There are so many variables. The establishment of an experimental method to induce tumor growths will provide an area in which therapeutic measures can be tested under controlled conditions.

Dr. Patz has determined the course of such tumors which will afford us the proper base line for future studies along these lines.

The lens has always been resistant to tumor stimulation. It will be interesting to note if his technique can produce neoplastic growth in this unique organ; also whether this technique will prove effective in the ocular tissue of experimental animals other than the special strains of mice that he has employed.

The field opened for study by Dr. Patz's observations are manifold. It will afford ocular oncologists an opportunity to catch up with those working in other areas of the body. We certainly are indebted to him for his present contribution and will look forward with great interest to the results of his future studies.

Dr. Leo L. Mayer (Jackson, Mississippi): I might shed a little light on this whole subject by telling you of an experiment that was done by Dr. Arthur Weil and myself. Dr. Weil was the neuropathologist at Northwestern University in 1933.

We produced retinoblastoma in mice. We injected the methylcholanthrene in superheated lard at that time because Dr. Weil had previously used this in the brain. We found that we did not have to use methylcholanthrene, that simply by using superheated lard we could produce retinoblastoma.

This sort of experiment has been done before, and I would not go along with Dr. Leopold that the

suture had anything to do with it. This was done simply by injecting with a hyperdermic needle; no sutures or anything else were used. This tumor took at least two years to produce. It was reported in the *Archives of Ophthalmology* in 1940.

DR. ARNALL PATZ (closing): I would like to thank the discussants for their comments.

I think the suture may possibly be a co-carcinogen because, when we look back on the figures for the tumor yield produced by simply putting the fused pellet or the crystals in alone, the tumor yield was about six percent. The tumor yield, when a cholanthrene suture was introduced, was approximately 10 percent. So this might reflect the enhanced carcinogenic effect of the suture itself.

I was aware of Dr. Wiel's and Dr. Mayer's report, which appeared in the *Archives of Ophthalmology*. We have observed a type of lesion similar to that shown in the photomicrographs of their paper—when we introduced almost any type of foreign material into the vitreous and got a subsequent gliosis, folding, and apparent proliferation of the retina. I would be most interested in seeing the slides themselves from Dr. Weil's and Dr. Mayer's experiments, or seeing what further data have appeared from those original reports.

We have not attempted to introduce the crystal into the crystalline lens, which Dr. Leopold asked about, although inadvertently we have perforated the lens capsule, and I am sure the carcinogen was in contact with lens epithelium. We observed no lens tumors.

IMMUNOGENIC ENDOPHTHALMITIS PRODUCED IN THE GUINEA PIG BY DIFFERENT PATHOGENETIC MECHANISMS*

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Since the early demonstrations by Filatov[1] and Sattler[2] that uveitis might be induced by immunologic mechanisms, and since Wessely's[3] work on immunogenic keratitis, hypersensitivity reactions have received fairly wide recognition as potential causes of a number of ocular diseases. The rather extensive literature relating to the clinical and experimental observations in this area have been summarized elsewhere.[4-6] It has been postulated[5] that nongranulomatous uveitis in man is the result of a delayed hypersensitivity mechanism similar to that responsible for the tuberculin reaction. Indeed, the terms "bacterial allergy" and "microbiallergic process" have been applied to these conditions, implying not only a delayed hypersensitivity mechanism, but also something about the nature of the etiologic agent responsible for the disease.

The delayed hypersensitivity state and its manifestations have been identified with the response to bacterial infections largely because the condition was first recognized and has been most extensively studied in connection with tuberculosis; indeed the tuberculin reaction is generally considered its prototype. It should be pointed out, however, that most fungi and many viruses,[7] and even simple bland proteins such as egg albumin[8-10] are capable of eliciting the tuberculin-type of delayed hypersensitivity response.

In addition to the wide range of etiologic agents which may potentially produce acute uveitis or endophthalmitis, on the basis of a "microbiallergic" mechanism, it must be pointed out that other imunological mechanisms may operate to produce ocular disease. Whereas the delayed hypersensitivity mechanism appears not to be mediated by circulating antibody, uveitis has been produced in experimental animals with pure protein antigens by antibody-mediated mechanisms.[11] The results of our previous studies[12, 13] and of those described herein, provide additional support for this suggestion, since a considerable range of tissue responses have been obtained with the same antigenic materials given under different conditions. By varying the method of sensitization, route of administration and dose of antigen, the several immunological mech-

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anisms may be stimulated to produce ocular lesions. Thus, the "wheal and erythema" response, the Arthus phenomenon, the tuberculin-type hypersensitivity reaction, and even local antibody production may severally or in concert contribute to the histopathologic picture of an ocular lesion, often with clinical manifestations which seem to be identical.

The present experiments are offered to illustrate this point, wherein clinically similar conditions of nongranulomatous endophthalmitis have been produced in the guinea pig by simple protein antigens and mediated by different immunological mechanisms.

Materials and Methods

Guinea pigs

Young female adult albino guinea pigs of the Hartley strain weighing 300 to 450 gm. were used. They were fed purina rabbit chow supplemented with kale and carrots, with water ad lib. All eyes were examined for signs of abnormality before initiation of each experiment.

Antigens

The protein antigens employed were twice recrystallized egg albumin (Ea, Worthington Biochemical Co., Freehold, New Jersey, Lot #542) and bovine serum albumin (BSA, Nutritional Biochemicals Corp., Cleveland, Ohio, Lot #5896). The antigens were prepared as 1% solutions in isotonic saline, Seitz-filtered, and stored in aliquots at −20°C. without preservative. Immediately before use, the antigens were thawed and diluted as required with sterile saline. The same antigen was used for both sensitization and challenge in a given animal. An out-dated commercial preparation of BCG (kindly provided by the Research Foundation, Chicago, Lot #10 BLP) was employed for tuberculin sensitization of the guinea pigs in some experiments. The organisms were suspended in saline and boiled for 20 minutes prior to use. Purified protein derivative (PPD) of the tubercle bacillus (second test strength, Merck Sharp and Dohme, Lot #3757 G) was dissolved in sterile saline, rather than in the commercial preservative solution provided, and used immediately.

Sensitization of animals

Active Arthus sensitization was induced by the intraperitoneal injection of 1.0 mg. of protein in 0.1 to 0.5 ml. of sterile saline. The animals were challenged 12 to 14 days later, at which time circulating antibody could be demonstrated; control animals prepared in the same way could consistently be sent into acute, usually fatal, anaphylactic shock upon intravenous challenge with 1.0 mg. of antigen. Passive Arthus sensitization was achieved by the intraperitoneal injection of suitable volume of either rabbit anti-Ea, or rabbit anti-BSA containing 1.0 mg. of antibody nitrogen. The rabbit antisera were obtained following repeated intramuscular injections of the antigen in combination with Freund's adjuvant.[14] The passively Arthus-sensitized guinea pigs were challenged by intravitreal injections of antigen two hours after sensitization.

A state of delayed hypersensitivity to tuberculin was initiated by injection of a total dose of 2.0 mg. of heat-killed BCG incorporated as in a Freund adjuvant.[14] The tuberculin-sensitive animals were challenged two weeks later. A state of delayed hypersensitivity to simple protein antigens was achieved by the method of Uhr, Salvin and Pappenheimer.[9] In this procedure, a total of 3.0 μg. of either Ea or BSA was used in the form of an immune precipitate prepared in the region of antibody excess (four-fold more antibody than present at equivalence), incorporated in a complete Freund's adjuvant, including myco-bacteria, and distributed into the four foot pads of the guinea pig. The animals so prepared were challenged 12 to 14 days following sensitization. At this time, as was found by Uhr and coworkers,[9] acute anaphylactic shock could not be elicited by intravenous challenge with antigen, nor could antibody be demonstrated in the serum by the extremely sensitive pas-

sive cutaneous anaphylaxis test. The intradermal skin test reaction at this time in control animals was typical of the delayed response as described by Uhr, et al.[9]

Challenge injections

All intraocular challenges with antigen were made into the vitreous as described previously.[12] The guinea pigs were anesthetized lightly with ether. Since the guinea pig's lens is larger in proportion to the vitreous body than is that of the rabbit, the hypodermic needle as it passes through the pars plana must be angled back somewhat more to avoid perforating the posterior lens capsule. Whereas 0.1 ml. could be injected with no difficulty into the rabbit vitreous, a maximum of 0.03 ml. of solution containing the required amount of antigen was injected into the guinea pig vitreous. Skin tests were performed in control animals by injection of 0.05 ml. of antigen intradermally in the preshaved ventral area.

Observations

The eyes were examined at suitable intervals after challenge. The more severe responses, consisting of chemosis, perilimbal injection, precipitates and fibrin in the anterior chamber, distortions of the pupil produced by posterior synechias, and diffuse keratitis were recognizable to the unaided eye. The slitlamp and ophthalmoscope were employed as required to detect less blatant changes. Representative eyes were enucleated at intervals after challenge for histologic examination, two or more eyes being taken for each period. These were fixed in aqueous formalin, embedded in paraffin, sectioned at 5.0 to 7.0 microns thickness, and stained with hematoxylin and eosin.

Results

A. Response of guinea pigs rendered delayed-hypersensitive to protein antigens

Guinea pigs were sensitized by the injection of antigen-antibody precipitates in Freund's adjuvant, by the method of Uhr, Salvin, and Pappenheimer.[9] Both Ea and BSA were used, in different experiments. The experimental observations were consistent with both antigens, and the results will be discussed together.

Intravitreal challenges with 3.0 μg. of antigen were made 12 to 14 days after sensitization. At this time, acute anaphylactic shock could not be elicited in comparably sensitized control animals, nor could circulating antibody be demonstrated by the extremely sensitive passive cutaneous anaphylaxis technique[15] or the tanned-cell hemagglutination technic.[16] Skin testing at this time produced the characteristic delayed-type hypersensitivity response, typical of animals sensitized in this manner.[9]

The early response to intravitreal injection of antigen in animals of this group was rather consistent. At 15 minutes there was a mild dilatation of vessels at the limbus and a slight haziness of the vitreous. Microscopically the posterior segment and anterior uveal tract appeared normal while a few polymorphonuclear leukocytes were present around the dilated limbal vessels. At one hour, there was slightly more response at the limbus, with scattered polymorphonuclears infiltrating the peripheral cornea.

Four hours following challenge, microscopic alterations were more obvious although clinically only a mild hyperemia of the iris and a somewhat more marked vascular dilatation at the limbus were observed. More lymphocytes and monocytes were present at the limbus, while polymorphonuclears and a few lymphocytes diffusely infiltrated the peripheral cornea. The iris, especially at its root, contained monocytes and large undifferentiated mesenchymal cells. A mild perivascular round cell infiltrate was also present at the optic nervehead.

The ocular reactions 24 hours after challenge were remarkably severe, both clinically and histologically. In the more intensely involved eyes, the extent of the keratitis was such that clinical examination of the intraocular tissues was impossible. Mild to moderate chemosis was present in some animals.

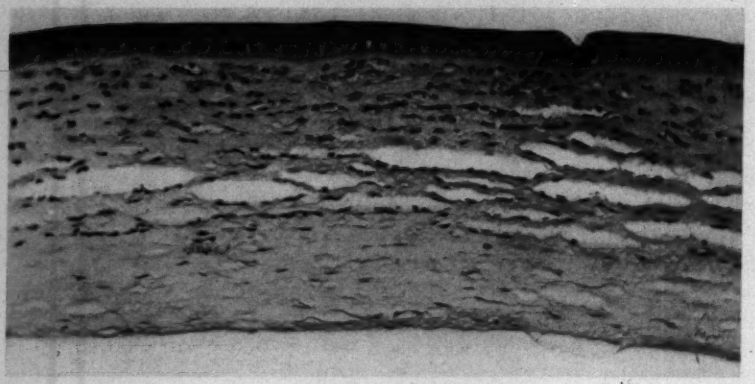

Fig. 1 (Silverstein and Zimmerman). Diffuse polymorphonuclear leukocytic infiltration is observed in the corneal stroma of delayed hypersensitive guinea pig 1 J 24 hours after intravitreal challenge with BSA. (Hematoxylin-eosin, ×165.)

When the cornea was less cloudy, the iris appeared markedly hyperemic, the anterior chamber contained much proteinaceous exudate, cells, and occasional fibrin strands, and the vitreous was cloudy, obscuring the fundus.

Microscopically, the limbus was intensely infiltrated with mononuclear and polymorphonuclear cells, while the cornea was massively infiltrated with polymorphonuclears (fig. 1). A scattering of large mononuclear elements was also present in the cornea. Capillaries could be seen as far as half way in toward the center of the cornea. In the iris and ciliary body, there was an extensive, diffuse infiltration (fig. 2), predominantly by large undifferentiated mononuclear cells, monocytes and lymphocytes (fig. 3). There was a sprinkling of polymorphonuclears in the iris stroma, and somewhat greater numbers in the ciliary body, particularly in the pars plana. There was a fibrinous deposit on the anterior iris surface. In one of the eyes examined at this time, there were a few small petechial hemorrhages in the iris. The anterior chamber contained both round cells and polymorphonuclears, as well as a proteinaceous exudate. The same cells were seen in the posterior chamber and anterior vitreous. There was some infiltration, predominantly by round cells, of the preretinal vessels in and around the optic disc. In one eye the choroid had a few focal infiltrates of small mononuclear cells; in another there was a very heavy diffuse choroidal infiltration predominantly of large mononuclear cells but with some polymorphonuclears. In the subjacent sclera and episclera of this eye there was a pronounced infiltration of the same inflammatory cells, primarily about blood vessels (fig. 4).

The clinical and histologic picture of these lesions at 48 hours was essentially the same as was seen at 24 hours. There was then less proteinaceous exudate in the anterior chamber and the proportion of polymorphonuclear cells in the iris, anterior chamber, and posterior segment was reduced. Except for the corneal infiltrate, the cells elsewhere were chiefly undifferentiated mononuclear elements and macrophages.

By the third and fourth day, the corneal reaction had subsided somewhat, although there was still a mild diffuse polymorphonuclear infiltration throughout the cornea, involving both the superficial and deep lamellae. There was a relative absence of polymorphonuclear cells in the other tissues; a suggestion of early differentiation into plasma cells was noted but only an occasional fully acceptable mature plasma cell was seen.

Fig. 2 (Silverstein and Zimmerman). While the corneal stroma is diffusely invaded by polymorphonuclears, the iris and ciliary body are massively infiltrated by mononuclear inflammatory cells. From same eye as in Figure 1. (Hematoxylin-eosin, ×50.)

In the following week to 10 days, the reactions in the challenged eyes slowly subsided. Except in the cornea, polymorphonuclear cells had largely disappeared. The round cell response gradually waned and by the 6th day, mature plasma cells were in evidence. The keratitis was mild and patchy and had almost disappeared histologically by the 13th day. At this time, only a mild diffuse infiltration of lymphocytes, undifferentiated mononuclears, and immature and mature plasma cells, the latter the predominant type,

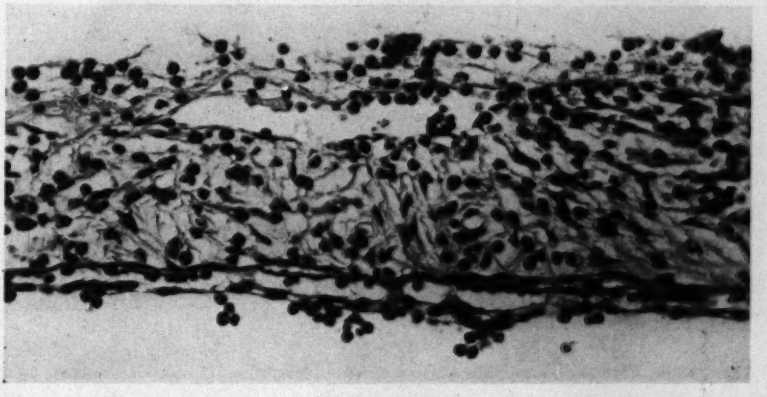

Fig. 3 (Silverstein and Zimmerman). The mononuclear infiltration of the iris shown in Figure 2 is illustrated at greater magnification. (Hematoxylin-eosin, ×305.)

was present at the limbus, in the iris and in the ciliary body, especially in the pars plana (fig. 5).

B. Response of guinea pigs rendered tuberculin-hypersensitive

Fourteen days after sensitization with heat-killed B.C.G., intravitreal injections of 0.5 μg. of PPD were made to serve as controls for the ocular delayed-hypersensitivity response elicited with protein antigens. The ocular tuberculin responses, and the other immunogenic reactions described below were followed only for the first few days, in order to compare the acute phase of the clinical and histologic reactions with those described above.

Clinically, the responses were similar to those observed following protein antigen challenge of delayed-hypersensitive guinea pigs. There was a minimal reaction in the first few hours, while at 24 hours there was in some eyes an intense keratitis obscuring internal observation, with varying degrees of chemosis. When the interior could be visualized, there was marked iritis with cells, proteinaceous exudate and occasionally fibrin in the anterior chamber.

Histologically, during the first few days, the picture appeared to be of the same qualitative nature as in the animals rendered delayed-hypersensitive to protein antigens. The only noteworthy exceptions were that a larger proportion, but not all, of the tuberculin-challenged eyes had petechial hemorrhages in the iris stroma, and that the proportion of polymorphonuclear leukocytes was somewhat greater than in the guinea pigs which were delayed-hypersensitive to protein antigens. The cellular response within the uveal tissues, however, was still predominantly mononuclear.

C. Response of active Arthus-sensitized guinea pigs.

Two weeks after intraperitoneal sensitization with 1.0 mg. of BSA, the guinea pigs were challenged into one eye only with either 50 μg. or 300 μg. of the same antigen. Both the clinical and histologic responses to the

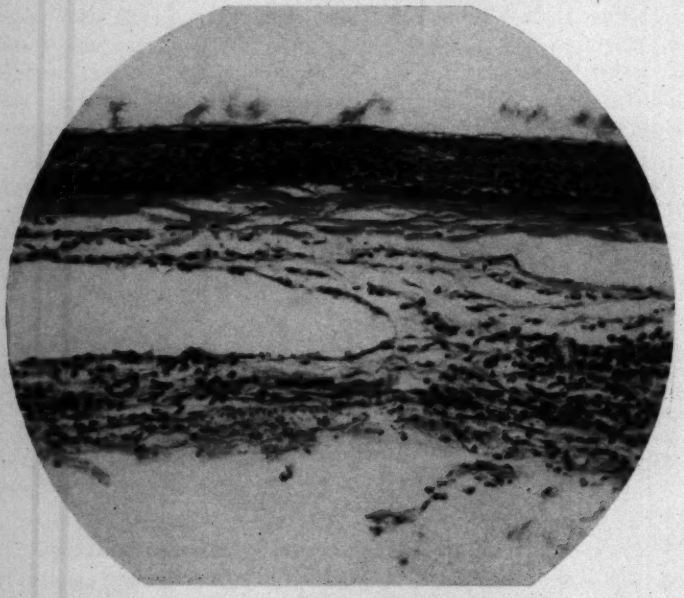

Fig. 4 (Silverstein and Zimmerman). The same guinea pig eye shown in Figures 1 to 3 exhibits a predominantly mononuclear infiltrate in the choroid, sclera, and episclera. (Hematoxylin-eosin, ×220.)

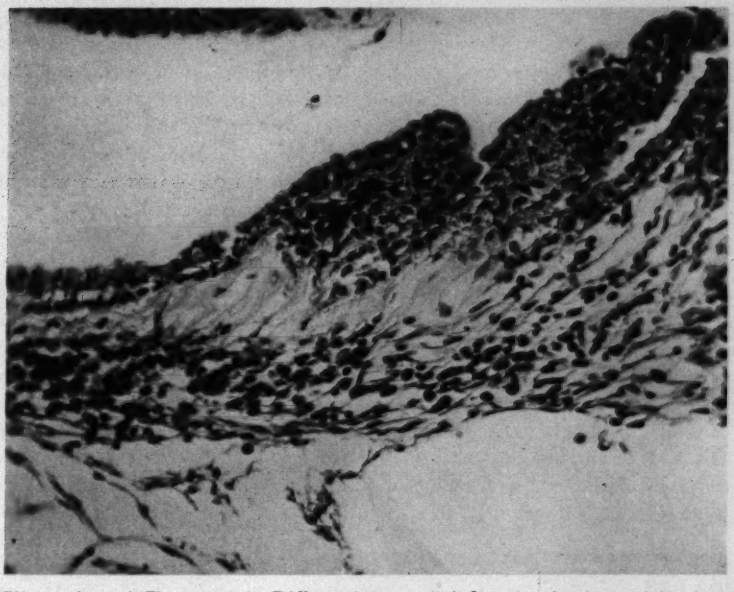

Fig. 5 (Silverstein and Zimmerman). Diffuse plasma cell infiltration is observed in the ciliary body of delayed-hypersensitive guinea pig 7A 13 days after intravitreal challenge with BSA. (Hematoxylin-eosin, ×300.)

lower dose were more mild and more transient than after the larger dose. The response to 50 μg. was characterized at three hours by a mild injection of limbal vessels about which was a light infiltrate of round cells and polymorphonuclear leukocytes. The iris and choroid were only slightly infiltrated by the same cells and the posterior segment was generally uninvolved. In one eye only at this time were there moderately intense focal choroidal infiltrates of mononuclear and polymorphonuclear cells, associated with a perivascular inflammatory infiltrate in the posterior orbital tissue. At 24 hours, the limbal reaction was only slightly more pronounced, as was the anterior uveal response. Polymorphonuclears and capillaries infiltrated the peripheral cornea for only a short distance. There was a moderate round cell infiltrate around the preretinal vessels about the optic disc, and a mixed polymorphonuclear and round cell infiltrate in the posterior orbital tissue.

In contrast to the response to 50 μg. of antigen, the injection of 300 μg. produced a much more violent reaction. While the picture at three hours was not appreciably more intense, that at 24 hours was more marked. In some of the animals at this later time there was marked chemosis, and severe keratitis usually made internal examination impossible. When the anterior segment could be viewed, marked iritis and a few petechial hemorrhages of the iris were observed in some eyes. Proteinaceous exudate, cells and often fibrinous strands were evident in the anterior chamber, while keratic precipitates and material on the anterior lens surface were also visible.

Microscopically, the thickened cornea was diffusely infiltrated, primarily by polymorphonuclear cells, while at the limbus there were both round cells and polymorphonuclears, the latter predominating. The iris and ciliary body were massively infiltrated, mostly by polymorphonuclears, but with round cells in evidence. The ciliary body, especially in the pars plana, was more involved than the iris. Petechial hemorrhage in the iris was seen occasionally. The anterior chamber contained many cells and a pro-

teinaceous exudate, as did the posterior chamber and anterior vitreous. There was a minimal cellular infiltrate at the optic disc. In one eye there were focal infiltrates of round cells and polymorphonuclears in the choroid, as well as a light infiltrate in the posterior orbital tissue adjacent to the optic nerve. In those eyes which had shown mild response, the corneal infiltration was less pronounced, and in some eyes limited to the periphery. Also, in these eyes, the cells in the anterior chamber, vitreous, and infiltrating the iris and ciliary body were fewer, but the cell types were the same.

The clinical and histopathologic picture of the response 48 hours after challenge in these animals had essentially the same features as those at 24 hours, and will not therefore be described further.

D. Response of passive Arthus-sensitized guinea pigs

Two hours after intraperitoneal injection of 1.0 mg. of antibody nitrogen (Rabbit anti-BSA) one eye of each animal was challenged by injection into the vitreous of 30 μg of BSA. This experiment served as a control for study of the active Arthus lesions. The smaller challenging dose chosen for this experiment was found to be adequate in producing a severe inflammatory response, presumably because the titer of circulating antibody in the passively sensitized guinea pig was higher than that which followed active sensitization.

The clinical course following antigenic challenge in these eyes was almost identical to that in the more strongly reactive intraperitoneally sensitized animals. At three hours there was mild chemosis and limbal injection but little intraocular response. At 24 hours there was a well developed keratitis (fig. 6), severe chemosis and intense iridocyclitis (fig. 7). Petechial hemorrhages in the iris were more frequently visible in this group than in any of the other experiments. Microscopically, the overall picture was not dissimilar quantitatively from that seen in the active Arthus lesions. However, in all of the passive lesions the polymorphonuclear leukocyte was more abundantly evident, round cells comprising only a very minor part of the histologic picture. As in the case of the active Arthus lesions, identi-

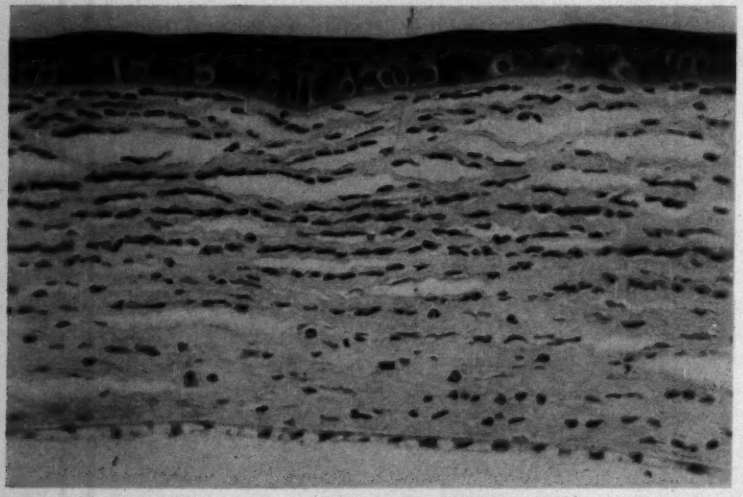

Fig. 6 (Silverstein and Zimmerman). Diffuse polymorphonuclear leukocytic infiltration is observed in the corneal stroma of the passively Arthus-sensitized guinea pig Y-2 24 hours after intravitreal challenge with BSA. Observe the similarity to the corneal infiltration found in the delayed-hypersensitive guinea pig shown in Figure 1. (Hematoxylin-eosin, ×220.)

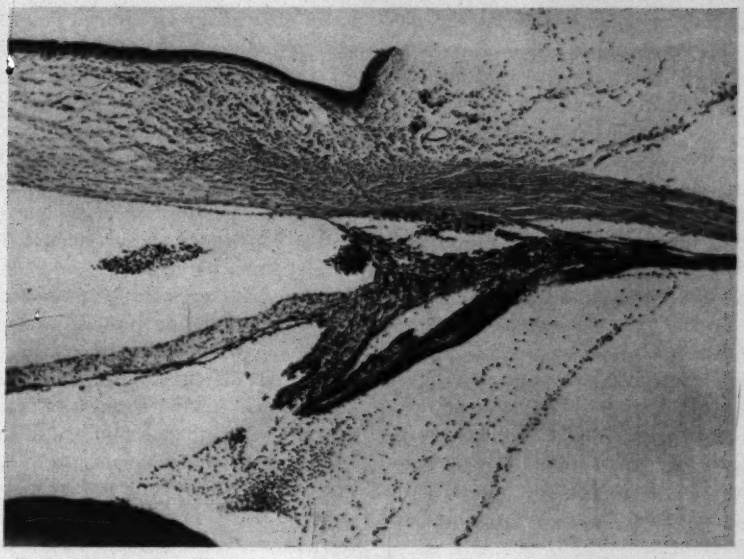

Fig. 7 (Silverstein and Zimmerman). The same guinea pig eye shown in Figure 6 exhibits an intense exudative iridocyclitis which differs from that shown in Figure 2 in that it is characterized almost solely by polymorphonuclear leukocytes. (Hematoxylin-eosin ×50.)

fiable thrombosis was generally absent in these eyes.

Discussion

The role of immunological hypersensitivity mechanisms in the production of ocular disease has been considered since early in the present century.[1-3] It has not always been clear, however, which of the immunological mechanisms known to cause inflammatory lesions might contribute to the pathogenesis of a given disease process. This has been especially true of immunogenic uveitis and endophthalmitis. Even less is known with certainty of the nature of the etiologic agents which stimulate these hypersensitivity conditions.

A mechanism of delayed bacterial hypersensitivity, akin to that giving rise to the tuberculin reaction, has been suggested[5] as the factor responsible for the inflammations of nongranulomatous uveitis in man. This immunologic process has been characterized by its lack of dependence upon conventional circulating antibody and by the temporal delay in the inflammatory response upon intradermal skin test challenge as usually performed (other characteristics of this process are discussed at length elsewhere).[7, 17] Since delayed hypersensitivity has classically been identified with bacterial infections and bacterial antigens, it was also postulated that the etiologic agents responsible for these cases of acute, sterile uveitis were bacterial in origin. The process is indeed often referred to as "microbiallergic."

Waksman and Bullington[11] have shown that a condition similar to acute nongranulomatous uveitis may be induced by simple protein antigens in their study of the passive Arthus reaction in the rabbit eye. In this case, evolution of the lesions was unequivocally based upon the interaction of protein antigen with circulating antibody, since the sensitization was achieved passively with hyperimmune antiserum.

We have extended the observations on the production of immunogenic endophthalmitis with simple, bland protein antigens, on the one hand by a mechanism involving the

Arthus phenomenon and mediated by circulating antibody, and on the other hand by a delayed-hypersensitivity mechanism. Guinea pigs made delayed-hypersensitive to Ea or BSA were challenged with small doses of the homologous antigen (3.0 μg.) injected into the vitreous. Animals rendered tuberculin-sensitive were used for comparison. During the first few days, the clinical and histologic responses in the two groups of delayed-hypersensitive animals were qualitatively and often quantitatively indistinguishable, both starting within a few hours, and developing to a peak at 24 to 48 hours. In both instances, the histologic reaction was predominantly a mononuclear infiltration of the ocular tissues, with polymorphonuclears playing a minor role. Only in the cornea was the polymorphonuclear leukocyte the principal cell type.

In guinea pigs rendered actively sensitive by intraperitoneal injection of protein antigen, or passively sensitive by injection of hyperimmune serum containing antibody, the clinical responses were essentially similar to one another, and also to those observed in the delayed-hypersensitive animals. Again, there were varying degrees of diffuse keratitis and the typical picture of an acute nongranulomatous iridocyclitis with inflammatory involvement at the optic disc. While petechial hemorrhage was a somewhat more consistent clinical finding in the Arthus-sensitized animals, its absence in many of these and its presence in the occasional delayed-hypersensitive animal deprived it of diagnostic significance.

We feel that with a more adequate control of the degree of sensitization of the animals in the different groups, severe iritis without any hemorrhage at all could have been achieved in every case. Even the temporal development of the lesions did not provide a basis for choosing between the mechanisms involved. In all cases the reaction started only after several hours, reaching an apparent peak of response at 24 to 48 hours. The initial lag may be ascribable to the slow escape of antigen from the vitreous,[11, 13] while the similarity in timing of the maximum reaction has often been described in response to skin testing of animals sensitized by these techniques.

Only histologically was it possible to differentiate the delayed-hypersensitivity response and the antibody-mediated reactions. It was consistently observed that the mononuclear cell characterized the responses of the delayed-hypersensitive guinea pigs, both to PPD and to the protein antigens. Contrariwise, the polymorphonuclear leukocyte played the dominant role in both the active and passive Arthus responses. It is significant, however, that in the case of the active-Arthus sensitized animals, the polymorphonuclear response appeared to be superimposed upon a mononuclear component. In the passive Arthus lesions,* while there were some round cells in evidence, these were relatively few. Gell and Hinde[18] and Gell (ref. 17) have suggested that the mononuclear-response is typical of the delayed hypersensitivity lesion, while the polymorphonuclear inflammation is seen in the acute passive Arthus reaction. They interpret the mixed response seen in the active Arthus situation as a superposition of the polymorphonuclear component elicited by antigen-antibody interaction upon a delayed hypersensitivity component, characterized by the presence of mononuclear elements. Thus, they suggest that the lesion in the actively sensitized animal may be pathogenetically a complex of responses.

It may be significant that the subsiding reaction in those animals rendered delayed-hypersensitive to protein antigens is one of extensive plasma cell differentiation. This,

* While we experienced no difficulty in inducing ocular inflammation in passively sensitized guinea pigs, as described briefly in this report, Waksman and Bullington[31] in their study of the passive Arthus response in the rabbit eye, indicated that they were unable to produce this phenomenon in the guinea pig. A more extensive investigation of the passive Arthus reaction in the guinea pig eye is in progress and will be described in a subsequent paper.

in all probability, is indicative of an appreciable level of local antibody production. While the active Arthus lesion results also in a wholesale differentiation to plasma cells, Gell[17] points out that this is not observed in bacterial hypersensitivity lesions, such as the tuberculin reaction, in which case there seems to be a block in the differentiation of mesenchymal cells to plasma cells. There is only very seldom the opportunity to examine human ocular tissues in the early phases of nongranulomatous uveitis, but we have observed in the later stages of these inflammations that there characteristically is an intense diffuse infiltration of the iris and ciliary body by plasma cells.

Another point of interest in the present study is the observation that a diffuse keratitis may accompany uveitis produced by the antibody-mediated Arthus mechanism as well as by a delayed hypersensitivity mechanism (figs. 1 and 6). The relatively early appearance of this response, as well as its massiveness, suggest that it is a primary manifestation and not secondary to the inflammatory process in the uveal tract. This observation is surprising since it has long been assumed[10] that immunogenic keratitis in the avascular cornea could be induced only in response to antigenic challenge in a delayed-hypersensitive animal.

Summary

1. Endophthalmitis has been produced in the guinea pig by two distinct immunologic mechanisms: (a) delayed hypersensitivity and (b) antigen interaction with circulating antibody. There appears to be no clear cut basis for distinguishing clinically the ocular inflammations produced by these two pathogenetic mechanisms.

2. Acute nongranulomatous uveitis due to a state of delayed-hypersensitivity may result, not only from "microbiallergic" processes involving bacterial antigens, but also as a consequence of tuberculin-type hypersensitivity to such simple bland protein antigens as crystalline egg albumin or bovine serum albumin.

3. Some of the immunologic and ophthalmologic implications of these experiments have been discussed.

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Acknowledgment

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Discussion

Dr. A. E. Maumenee (Baltimore): It gives me great pleasure to open the discussion on this paper, first because it was a most interesting presentation and, more important than that, it gives me the opportunity to welcome Dr. Silverstein to our group of ophthalmologists.

As all of you know, in the past years Dr. Zimmerman has been a general pathologist in charge of eye pathology at the Armed Forces Institute of Pathology and has contributed a great deal to our knowledge in this field. I am delighted the Chief of the Immunochemistry Branch of the AFIP, Dr. Silverstein, has become interested in this most complicated problem of uveitis.

I think this paper brings up several very interesting points that might be mentioned at this time and might be opened to further study. First of all, it is very interesting to me that, in spite of the fact that these antigens were injected into the vitreous, the primary reaction in these eyes was in the anterior segment.

This is very reminiscent of the nongranulomatous type of iridocyclitis that we see frequently, where the patients repeatedly have a violent attack of anterior uveitis with relatively little involvement in the posterior part of the eye.

One point in these studies that does not follow very closely the clinical appearance of such lesions, however, is the very marked involvement of the cornea in these guinea pigs. It is entirely possible that the guinea pig cornea differs in some way from human cornea to make this reaction so much more prominent.

Another thing that is interesting about this study, and that was not mentioned in the presentation but is mentioned in the paper, is that in some of the guinea pigs within a matter of 24 hours after the vitreous had been injected there is vascularization extending to the midportion of the corneal stroma. This seems to me to be an unusually rapid development of blood vessels in the cornea, and I wonder if it is possible that somehow or other these blood vessels may have been overlooked prior to injection, or possibly the guinea pig might have some preform vessels that were occluded in the corneal stroma.

The next point is that this study reopens the whole question of the type of antigen that is necessary to produce a chronic nongranulomatous iridocyclitis, because if this work can be transferred to the human then it is possible that any antigen—a food antigen, a breakdown food product of any sort, a breakdown of cell—can produce a nongranulomatous iridocyclitis.

I was rather interested in the histology that was presented, and I would like to ask Dr. Silverstein if he feels he could determine, on purely histologic examination of the eye alone, whether the animal had been injected with material that was likely to produce a delayed hypersensitivity as contrasted to the immediate hypersensitivity.

I know he mentioned that there were slight differences, but I wonder if he would put Dr. Zimmerman or himself to the test of a blind study to determine whether they could really differentiate between these two types of reactions.

It is rather interesting that they did not see any epithelioid cell proliferation in the eye, as does occur in skin testing with tubercular protein. As I understand it, Dr. Uhr has found in his antigen-antibody complex a production of delayed hypersensitivity. I wonder if the eye reacts a little differently in this matter.

Finally, to come back again to the corneal reaction, we have found also that the avascular cornea is able to react and show a response to the immediate type of hypersensitivity, that is, injection of antigen into the cornea of an animal that has previously been sensitized to the immediate type of hypersensitivity. In the past this has been thought to be one of the differentiating points between immediate and delayed types of hypersensitivity, in that the avascular cornea would react to the delayed type of hypersensitivity but not to the immediate.

Dr. Arthur M. Silverstein (closing): I should like to thank Dr. Maumenee for his discussion of this paper, and to say that we did subject ourselves to the blindfold test with all of these guinea pigs. With respect to being able to evaluate between the antigen-antibody interaction on the one hand and the delayed hypersensitivity reaction on the other, I think we passed with flying colors—but only, I think, because we looked at eyes that had been taken out at a certain time. If we had looked at the eyes earlier, I think we could not have differentiated be-

tween them, that is, at the very onset of the reaction.

If we looked at the eyes later I think we could not have differentiated, because it is classically known, with respect to skin lesions following immediate or delayed hypersensitivity (the Arthus *vs.* the tuberculin type), that after three or four days it is often almost impossible to distinguish between these lesions, and in the eye I don't think we could, either. Indeed, we could not, with the limited material that we had. So, one has to look at the right time.

I don't think one can say that there is any hallmark or anything pathognomonic histologically about any of these lesions. This is certainly true if one takes a lesion from the beginning to the end. I think one may generalize that at a certain time the polymorphonuclear leukocyte typifies the antigen-antibody reaction, and the round-cell infiltrate typifies the delayed hypersensitivity reaction.

Certainly, with respect to the epithelioid cells, I don't believe this is a necessary adjunct to any delayed hypersensitivity reaction.

EXPERIMENTAL OCULAR HYPERSENSITIVITY*

HISTOPATHOLOGIC CHANGES OBSERVED IN RABBITS RECEIVING A SINGLE INJECTION OF ANTIGEN INTO THE VITREOUS

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There is an extensive literature dealing with various aspects of the experimental production of immunogenic inflammation in intraocular tissues. Much of the available data is summarized in a monograph by Foss.[1] With few exceptions the observations made in the course of these experiments were clinical and macroscopic. Slitlamp and ophthalmoscopic examinations have often been made but histopathologic studies have been few.[2]

The present paper reports histopathologic observations we have made during one phase of our study of immunogenic endophthalmitis. Elsewhere we have reported on some extensions of the clinical observations made by Foss and others in rabbits given single intravitreal injections of antigen.[3, 4] When a 1 mg. dose of crystalline egg albumin is used, clinical signs of an endophthalmitis appear six to eight days after intravitreal injection in all of the rabbits injected. Smaller dosages in the range of 0.2 to 0.5 mg. will produce similar results in some but not in all of the animals. When 0.1 mg. or less of the antigen is injected the eyes usually fail to show clinical signs of an inflammatory reaction. We have, however, observed histopathologic evidence of a response to the small doses (0.05 to 0.10 mg.) of antigen as well as to the larger dose (1.0 mg.). The tissue reactions observed in eyes receiving these doses of crystalline egg albumin will be described in this paper.

* From the Ophthalmic Pathology and Immunochemistry Branches, Armed Forces Institute of Pathology. Supported in part by a grant-in-aid from the Council for Research in Glaucoma and Allied Diseases, Alfred P. Sloan Foundation, Inc.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Albino New Zealand rabbits weighing 2.0 to 3.5 kg. were used. They were fed purina rabbit chow. All eyes were examined before use for the presence of pre-existing disease.

ANTIGEN

Two times recrystallized egg albumin (Worthington Biochemical Corp., Freehold, New Jersey lot #542) was employed for most of the experiments. This was prepared as a 1% solution in isotonic saline, sterilized by Seitz filtration, distributed in small aliquots and stored at −20°C. without preservative. Appropriate dilutions of the thawed solutions were then prepared immediately before use with steril saline.

INJECTIONS

The injections into the vitreous were made after anesthesia with 0.5 percent tetracaine

hydrochloride. The eye was fixed by gently grasping the sclera with forceps, and the eye rotated downward. A sharp 27-gauge needle was then inserted at a slight angle backward at a point about two mm. behind the corneoscleral junction, so that the needle path would go through the pars plana of the ciliary body. With a further slight angling of the needle backward, it was possible to avoid perforating the posterior lens capsule, and 0.1 ml. of solution was injected slowly into the center of the vitreous. The amount of material which exuded from the injection site after removal of the needle was slight, and on only two occasions was a transient subconjunctival bleb observed. In the present experiments the second eyes were not injected.

Observations

The eyes were examined daily. The more severe reactions, including perilimbal injection, iris hyperemia, precipitates and fibrin in the anterior chamber, and distortions in the pupillary margin of the iris due to posterior synechias were recognizable to the unaided eye. The slitlamp was also used to observe earlier and less marked changes in the cornea, iris and anterior chamber, and the ophthalmoscope for changes in the posterior segment. Representative eyes were enucleated at intervals ranging from three hours to 64 days after injection and used for histologic examination. Initially the formalin-fixed eyes were opened in the vertical plane in an effort to obtain sections containing the more abundant exudate usually present in the inferior angle of the anterior chamber. Subsequently the horizontal plane was utilized to better observe the formation of exudate along the inner surface of the retina. In the rabbit retina only the horizontal meridian is vascularized, the remainder is avascular. Thus in vertical sections the only retinal vessels observed are those at the optic nerve head. All eyes were fixed in aqueous formalin, imbedded in paraffin, sectioned at 5.0 to 7.0 microns thickness, and stained with hematoxylin and eosin.

Histopathologic Observations

A. Intravitreal injections of 1.0 mg. crystalline egg albumin

Rabbits were killed and the eyes examined macroscopically and microscopically at the following intervals after a single intravitreal injection of 1.0 mg. of crystalline egg albumin: 3 and 24 hours, 3, 5, 7, 8, 11, 13, 15, 21, 28, 35, 49, 57 and 64 days. One to five eyes were examined for each of these time intervals. Those rabbits killed before the seventh day had shown only a mild transient nonspecific reaction to the trauma of injection. Those obtained at seven and eight days had just begun to exhibit clinical manifestations of the specific inflammatory process. Those taken after the 11th to 13th day represent the subsiding clinical reaction.

Three hours. A small amount of proteinaceous exudate could be seen adhering to the corneal endothelium of the inoculated eye of one rabbit but not in that of the other. No inflammatory cells were observed in the anterior segment of either, but in one there were a very few polymorphonuclears and monocytes in the vitreous adjacent to the optic disc.

Twenty-four hours. An acute inflammatory reaction with thrombosed vessels was observed at the injection site in the sclera. Limbal hyperemia and the formation of an inflammatory pannus were evident in each of the two injected eyes and a very small amount of proteinaceous exudate was present in the aqueous of one. Very few inflammatory cells were seen in the anterior segment but some polymorphonuclears, lymphocytes and monocytes were present in the posterior vitreous along the retina and in front of the disc.

Three days. At the injection site of one animal there was still an acute inflammatory reaction with thrombosed vessels. The other rabbit's injected eye revealed a very mild limbal reaction with rare polymorphonu-

clears, lymphocytes, and plasma cells. An occasional blood vessel was observed in the peripheral cornea but there was no significant cellular infiltration. Rare lymphocytes and monocytes were present in the anterior and posterior chambers, adhering to the iris and to the ciliary processes. There were focal hemorrhages along the inner surface of the retina posteriorly and these were heavily infiltrated by polymorphonuclears and monocytes. The choroid was not remarkable.

Five days. Of the five injected eyes examined, one revealed no limbal reaction, one presented a mild inflammation, and in the other three there was a rather intense infiltration by tightly packed undifferentiated mononuclears and some polymorphonuclears which extended into the peripheral cornea as an inflammatory pannus. A few blood vessels and polymorphonuclears extended into the peripheral cornea from the limbus in four of these eyes. Occasional lymphocytes and monocytes were observed in the anterior chamber angle and attached to the iris surface.

In the two eyes which exhibited the most intense limbal and corneal reaction there were also mononuclear infiltrates in the iris stroma (fig.1). Some of these cells appeared to be lymphocytes with scanty or absent cytoplasm. Others presented a similar dark round nucleus eccentrically situated in a more abundant, basophilic cytoplasm suggestive of plasma cell differentiation. Still other cells presented monocytoid features.

The same two eyes which presented the iritic lesions also revealed moderately intense mononuclear infiltration of the vitreous. Again the same cell types were present, though in the vitreous there were greater numbers of large mononuclears, many of which were differentiating as macrophages. On the surface of the optic disc there was a moderately intense round cell infiltration (fig. 2); some immature and a few differentiated plasma cells were present.

Similar infiltrates in the vitreous along the inner surface of the retina were observed in the other three eyes but in these the reaction was minimal. No choroidal lesions were observed.

Seven days. Both of the injected eyes revealed an intense limbal reaction and formation of an inflammatory pannus (fig. 3-a). The limbal infiltrate was mononuclear with many plasma cells in various stages of differentiation. The peripheral cornea was invaded by blood vessels and infiltrated by polymorphonuclears. There were very few cells and no proteinaceous exudate in the anterior chamber.

The iris of one eye was heavily infiltrated by mononuclear cells while that of the other

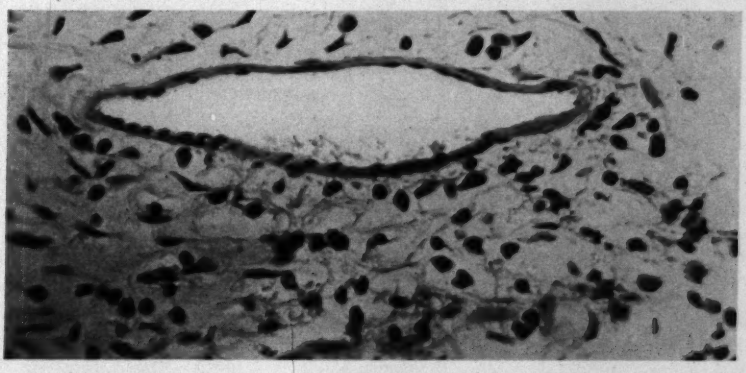

Fig. 1 (Zimmerman and Silverstein). Mononuclear infiltration in the iris—rabbit 26N, five days after intravitreal injection of 1.0 mg. crystalline egg albumin. (Hematoxylin-eosin ×450.)

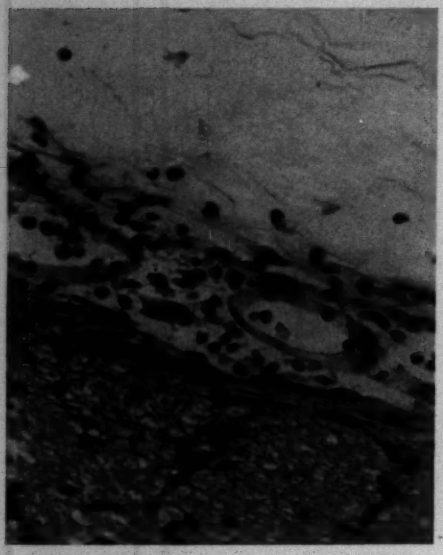

Fig. 2 (Zimmerman and Silverstein.) Mononuclear infiltration on surface of optic disc—rabbit 26N, five days after intravitreal injection of 1.0 mg. crystalline egg albumin. (Hematoxylin-eosin, ×380.)

injected eye was very lightly infiltrated. Where the mononuclear infiltration was most intense the iris stroma was thickened almost two-fold. The infiltrating mononuclear cells included both small round cells and large monocytoid and fibroblastic elements. Many were immature plasma cells and some well-differentiated plasma cells were also present. An occasional focus contained cells with bilobated nuclei and intensely eosinophilic cytoplasmic granules.

There was remarkably little reaction in the ciliary body and the anterior vitreous contained very few cells. Posteriorly there was a moderately intense mononuclear infiltration along the retinal vessels and over the surface of the optic disc (fig. 4-b). Considerable infiltration of the opsterior cortical vitreous was also observed. Large and small cells both contained hyperchromatic nuclei. Some were differentiating as macrophages while others presented features of plasma cells. These cells also infiltrated the inner-

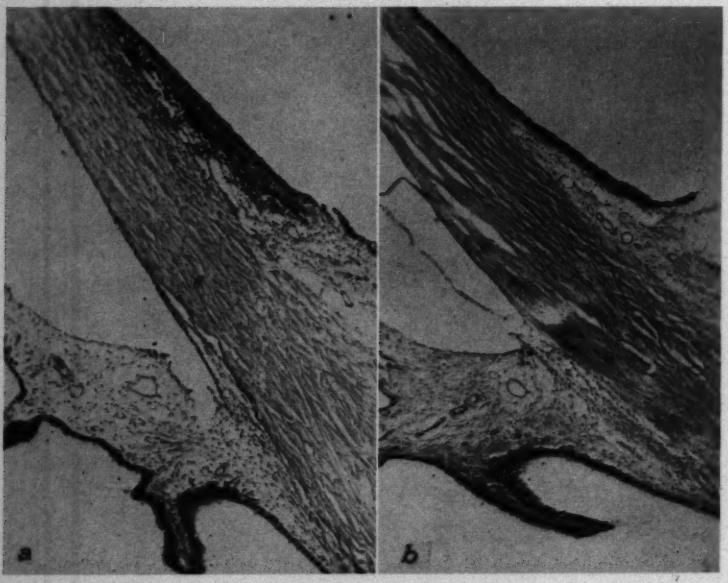

Fig. 3 (Zimmerman and Silverstein). (a) An intense mononuclear infiltration is observed at the limbus of rabbit 32N, seven days after intravitreal injection of 1.0 mg. crystalline egg albumin. (b) There is no significant reaction at the limbus of rabbit 36Q, seven days after intravitreal injection of 0.05 mg. crystalline egg albumin. (Hematoxylin-eosin, ×50.)

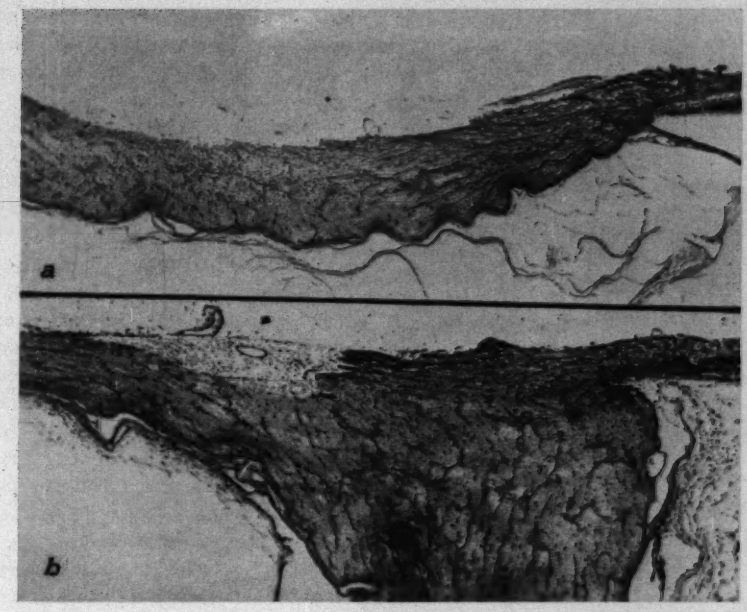

Fig. 4 (Zimmerman and Silverstein). (a) Very little reaction is observed at the optic disc of rabbit 36Q; (b) There is a moderate mononuclear infiltration on the surface of the optic disc of rabbit 32N. Same pair of rabbits shown in Figure 3. (Hematoxylin-eosin, ×50.)

most layers of the nerve head and adjacent retina. In one of the rabbits the orbital fat adjacent to the optic nerve contained many foci of lymphocytic infiltration.

Eight days. The only injected eye examined revealed a much more intense inflammatory reaction in all of the affected ocular tissues as compared with those from the seventh day. The corneal involvement extended further into the central area. Serosanguineous exudate filled the anterior chamber and organization of a blood clot was taking place in the inferior angle.

The iris was massively infiltrated by large hyperchromatic mononuclear cells, lymphocytes, and plasma cells in various stages of differentiation. In places the posterior layers of the iris were more intensely affected than the anterior and the inflammatory cells were migrating through the epithelium into the posterior chamber. Many clumps of these cells were present in the posterior chamber, on the lens capsule, in the zonule, and in the vitreous. The ciliary body was only slightly affected and the anterior choroid uninvolved.

Posteriorly there was very intense infiltration of the nervehead, adjacent retina and cortical vitreous. Moreover, there was a remarkable degree of choroidal thickening as a result of intense infiltration by tightly packed, hyperchromatic small mononuclear cells (fig. 5). Degeneration of the visual cells adjacent to this choroidal lesion was advanced.

Eleven days. All three of the injected eyes revealed an intense mononuclear limbal reaction with distinct evidence of plasmacytoid differentiation of the round cells. Capillaries, monocytes, and polymorphonuclears invaded the peripheral cornea. One eye revealed much serous exudate and some cells in the anterior chamber.

In all three the iris was diffusely infiltrated by mononuclear cells, many of which were plasma cells in varying stages of maturation (fig. 6), but a similar reaction was observed in the ciliary body of only one (fig. 7). This eye also revealed a more extensive

Fig. 5 (Zimmerman and Silverstein.) Intense mononuclear infiltration of the choroid—rabbit 75L, eight days after intravitreal injection of 1.0 mg. crystalline egg albumin. (Hematoxylin-eosin, ×305.)

migration of monocytes and lymphocytes into the vitreous anteriorly as well as posteriorly.

All three revealed many large and small mononuclear cells about vessels of the disc and retina. Some exhibited macrophagic or fibroblastic differentiation while others presented plasmacytoid features. The one eye which exhibited the more diffuse vitreal reaction also contained several unusually large aggregations of monocytic cells attached to the inner surface of the retina. One of these appeared to be forming a tuberculoid granuloma for there was a small central focus of necrosis and the surrounding cells were fusing and differentiating as epithelioid cells (fig. 8).

Special stains for fungi and bacteria, including gram and acid-fast stains, disclosed no microorganisms. Two of the eyes contained small focal round cell infiltrates in the choroid and in one of these there were small accumulations of serous exudate between the choroid and retina.

Thirteen days. The one injected eye examined on the 13th day revealed essentially the same changes as observed in the most markedly inflamed of the three eyes examined on the 11th day except for the absence of granulomatous nodules on the inner surface of the retina.

Fifteen days. The one injected eye examined revealed a subsiding limbal reaction but the anterior chamber was filled with serous exudate. The iris and ciliary body were somewhat less cellular but the cells present, particularly over the surface of the ciliary processes, were almost all mature plasma cells (fig. 9). In the vitreous and along the inner surface of the retina there were many large mononuclears and scattered lymphocytes but few plasma cells. A few agglutinated masses of large mononuclears, resembling the "mutton-fat keratic precipitates" observed in uveitis of man were attached to the retina. Several areas of round cell infiltration were observed in the choroid. These were associated with mild serous exudation beneath the retina and marked degeneration of the outer retinal layers.

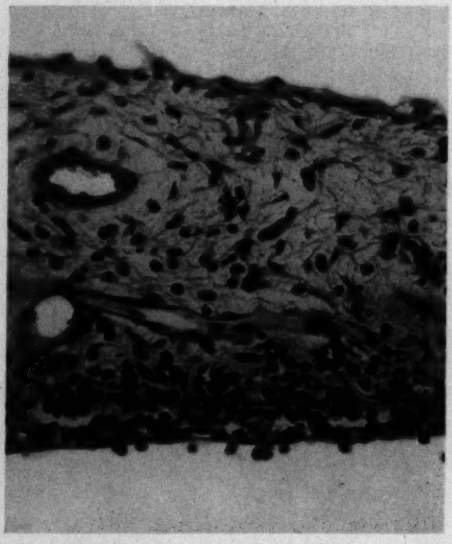

Fig. 6 (Zimmerman and Silverstein). Intense mononuclear infiltration of the iris with plasma cell differentiation, especially in the deeper layers—rabbit 61L, 11 days after intravitreal injection of 1.0 mg. crystalline egg albumin. (Hematoxylin-eosin ×305.)

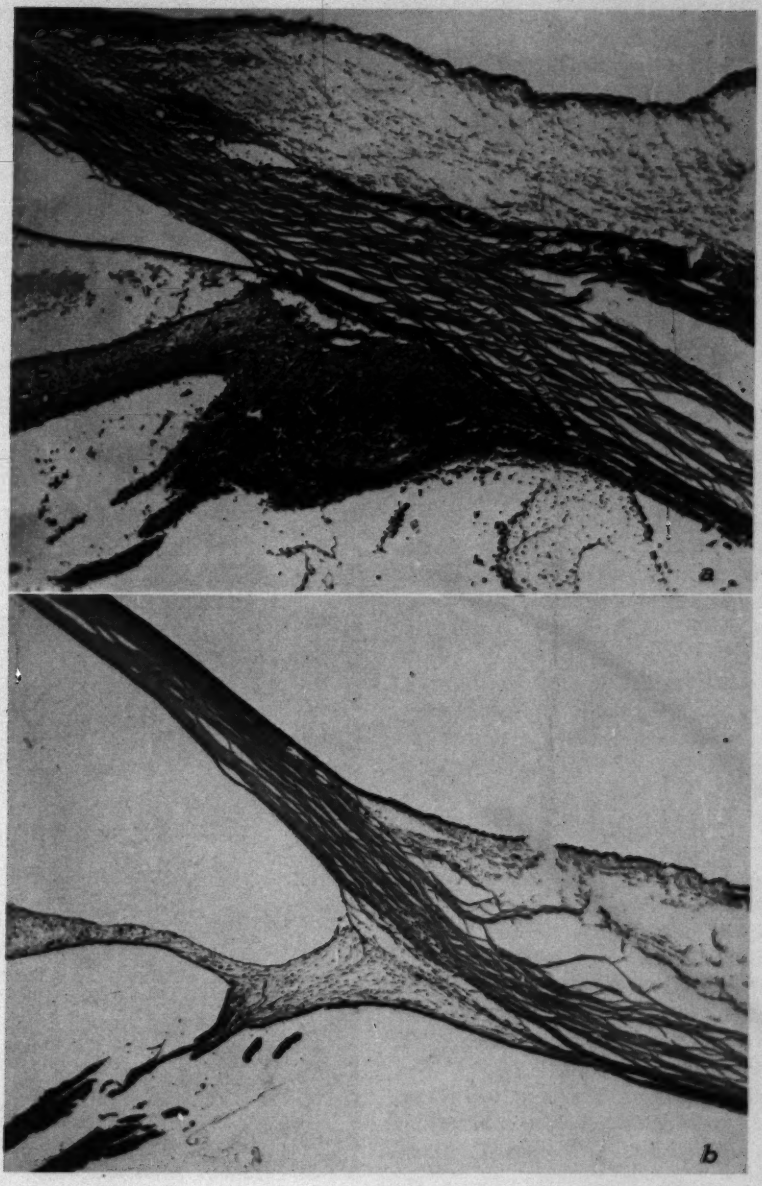

Fig. 7 (Zimmerman and Silverstein). (a) Massive mononuclear infiltration of ciliary body with exudation into vitreous and anterior and posterior chambers; from same eye shown in Figure 6. (b) Uninjected control eye from same rabbit reveals normal ciliary body. (Hematoxylin-eosin, ×50.)

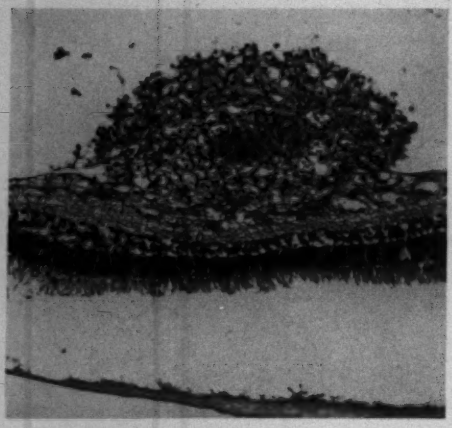

Fig. 8 (Zimmerman and Silverstein). Early granulomatous lesion has formed on the inner surface of the retina; from same eye shown in Figures 6 and 7-a. (Hematoxylin-eosin, ×145.)

Twenty-one days. The one injected eye revealed almost complete subsidence of the limbal reaction though the anterior chamber was filled with serous exudate. Only a very few mononuclear cells, chiefly plasma cells, were observed in the iris and ciliary body. A greater concentration of these cells covered the ciliary processes and infiltrated the vitreous base from the pars plana. Deeper in the vitreous the cells were mostly monocytes, macrophages, and lymphocytes. A great variety of small and large mononuclear cells covered the optic disc and adjacent retina. Included were some exhibiting fibroblastic differentiation and occasional mature plasma cells. Round cell infiltration was also observed in the parenchyma of the retina immediately beneath its inner surface about the disc. No choroidal infiltrates were observed but there was advanced denegeration of the visual cells, especially posteriorly.

Twenty-eight days. The only injected eye examined had exhibited very minimal inflammation clinically. Microscopically the anterior segment appeared virtually normal and no exudate was present in the anterior chamber. There was mild gliosis of the optic disc and its surface contained a light infiltrate of large and small mononuclear cells among which were a few plasma cells. Degeneration of visual cells was restricted to the retina immediately adjacent to the disc but no choroidal infiltrates were seen.

Thirty-five days. The one injected eye examined revealed a very light mononuclear infiltration, chiefly by plasma cells, in the limbus, iris, and ciliary body. Serous exudate occupied the anterior chamber. Mononuclear cells of all types, including plasma cells, lightly infiltrated the vitreous. An edematous vascular membrane infiltrated by plasma cells covered the retina about the disc. Contraction of this membrane produced fixed folds of the detached retina (fig. 10). A large accumulation of serous exudate occupied the subretinal space posterior to the equator. No choroidal lesions were observed.

Forty-nine days. The tissues of the anterior segment were essentially normal but collections of small and large mononuclear cells, including some plasma cells, were still

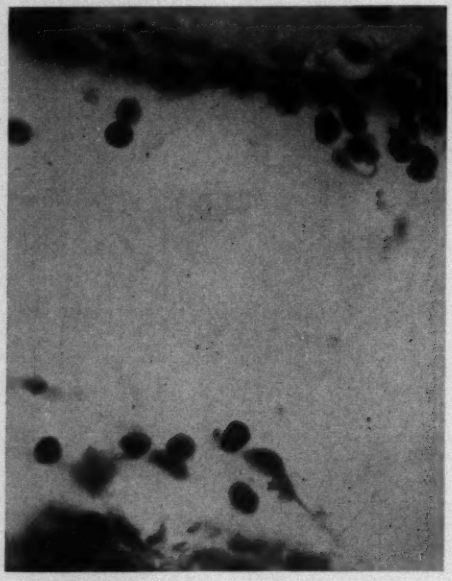

Fig. 9 (Zimmerman and Silverstein). Immature and mature plasma cells on the surface of the ciliary processes—rabbit 60L, 15 days after intravitreal injection of 1.0 mg. crystalline egg albumin. (Hematoxylin-eosin, ×450.)

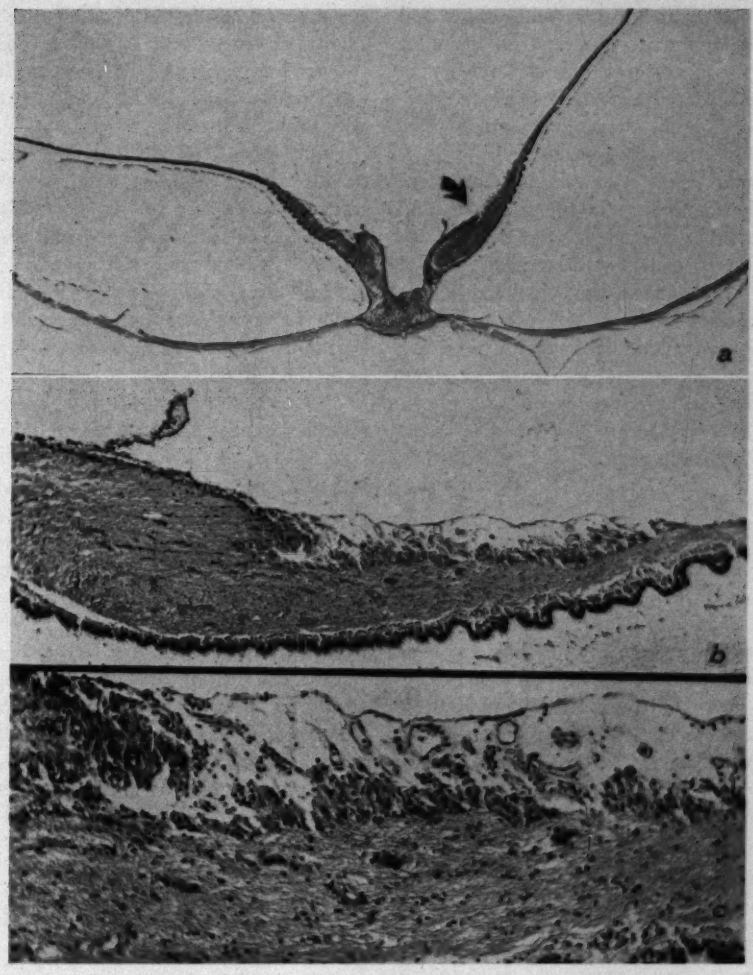

Fig. 10 (Zimmerman and Silverstein). (a) The retina is detached and there are fixed folds adjacent to the optic disc; the area indicated by the arrow is shown at greater magnifications in (b) and (c)—rabbit 52M, five weeks after intravitreal injection of 1.0 mg. crystalline egg albumin. (Hematoxylin-eosin, ×9, ×50, ×115, respectively.)

present in the vitreous. These same cells were present in very small numbers over the surface of this disc. The retina adjacent to the disc revealed moderate gliosis of the nerve fiber layer and degeneration of the visual cells. Occasional discrete foci of gliosis and round cell infiltration were also observed away from the nerve head. No choroidal infiltrates were present.

Fifty-seven days. Except for a rare round cell along the surface of the ciliary processes, the anterior segment of the one injected eye appeared uninflamed. There was a moderately intense perivascular round cell infiltration of the preretinal vessels and most of the inflammatory cells appeared to be small plasma cells. Fibrovascular and glial proliferation from the retinal surface into the

vitreous was evident in several places and similar changes were observed on the disc. Adjacent to the disc on one side the retina was folded inward and its outer layers exhibited severe degenerative changes. No choroidal infiltrates were observed.

Sixty-four days. Rare plasma cells were present at the limbus, in the iris root, and along the inner surface of the ciliary processes. The vitreous was clear. Perivascular round cell infiltration was observed in the retina, but this was light and not associated with as pronounced a degree of fibrovascular proliferation as was observed at 57 days. The retina exhibited only minimal gliosis and virtually no degeneration of its visual cells. No choroidal lesions were observed.

B. Intravitreal injections of 0.05-0.10 mg. crystalline egg albumin

Rabbits were killed and one or two pairs of eyes were taken for macroscopic and microscopic examination at the following intervals after a single intravitreal injection of 0.05 or 0.10 mg. crystalline egg albumin: 1, 2, 3, 4, 6, 7, 8, 11, 15, 18, 20, 23, and 30 days. Clinically these eyes were considered to be "nonreactors" except for the rabbit killed on the 20th day. This rabbit had shown a mild ciliary flush and aqueous flare beginning on the 17th day.

One day. There were a very few round cells at the limbus but the anterior segment and vitreous were otherwise free of inflammatory cells and exudate. The preretinal capillaries were engorged and small numbers of lymphocytes and monocytes were present immediately adjacent to these vessels. The loose connective tissues covering the optic disc appeared slightly more cellular than normal, the responsible cells being mostly lymphocytes.

Two days. There was an increase number of mononuclears, mostly lymphocytes at the limbus but only a rare monocyte was observed in the iris root. The anterior segment was otherwise not remarkable. A very few polymorphonuclears and monocytes were present in the vitreous. The preretinal hyperemia and exudation of mononuclears was slightly advanced than on the preceding day. A small amount of proteinaceous exudate was present beneath the retina in the mid periphery.

Three days. The changes observed in the tissues of the anterior segment were not significantly different from those noted earlier but posteriorly there was a complete absence of inflammatory reaction.

Four to six days. The tissues of the anterior and posterior segments revealed about the same degree of inflammatory reaction as on the first day.

Seven days. Almost all inflammatory cells had disappeared from all sites (figs. 3-b and 4-a).

Eight days. In one of the two eyes injected there was a mild diffuse increase in cellularity of the iris, especially in its deeper layers where a very light infiltrate of mononuclear cells was observed. In the other there was a mild exudation of mononuclear cells, some of which were moderately well-differentiated plasma cells, along the preretinal capillaries.

Eleven days. There was no significant inflammatory reaction anteriorly. Posteriorly the preretinal capillaries were markedly congested but there was minimal exudation of inflammatory cells. The vitreous and connective tissue in front of the disc were remarkably free of cells.

Fifteen days. The anterior segment and the vitreous were generally free of inflammatory reaction (fig. 11-a). The only significant increase in inflammatory cells as compared with earlier stages was observed in front of the nervehead (fig. 11-b). Here there was a moderately dense infiltrate of mononuclear cells, mainly in the connective tissue in front of the disc. Lymphocytes and monocytes predominated but there were also many immature and some mature plasma cells and fibroblasts (fig. 12). Some capillary proliferation was also evident. The tissues of the nervehead were slightly infiltrated by round cells. No choroidal infiltrates were observed.

Eighteen days. There were very few

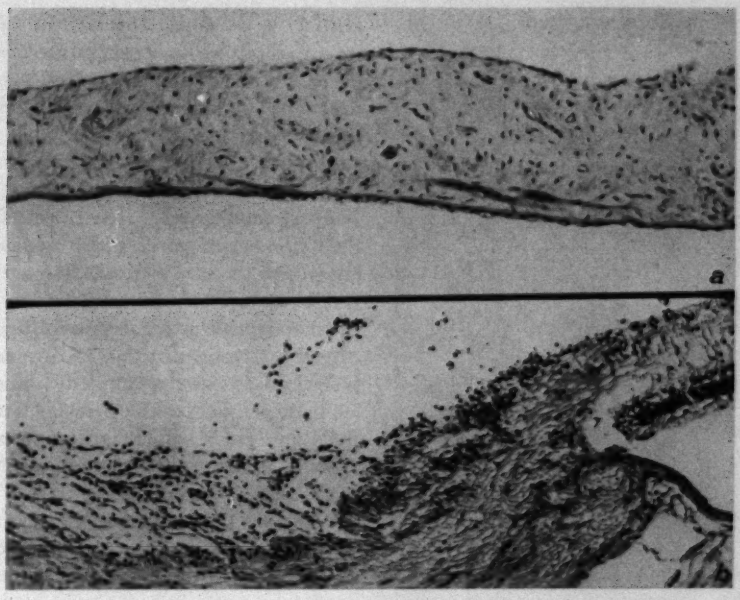

Fig. 11 (Zimmerman and Silverstein). (a) There is no significant infiltration of inflammatory cells in the iris. (b) A mononuclear infiltrate is present on the anterior surface of the optic disc. Both fields from rabbit 17P, 15 days after intravitreal injection of 0.10 mg. cyrstalline egg albumin. (Hematoxylin-eosin, (a) ×80, (b) ×110.)

round cells in the limbal tissues and the iris was not remarkable. The ciliary body on one side contained a moderate number of lymphocytes and plasma cells; a few monocytes and lymphocytes were present in the adjacent vitreous base. Posteriorly the inflammatory reaction was not significantly different from that observed on the 15th day, except for a moderate number of polymorphonuclears in the connective tissue surrounding the central retinal vessels. The preretinal infiltrates did not contain polymorphonuclears.

Twenty days. This eye obtained from the only clinically reactive rabbit in the low-dose series revealed an intense inflammatory reaction involving most of the tissues and chambers of the eye anteriorly and posteriorly. The limbal tissues were heavily infiltrated with lymphocytes and plasma cells.

An inflammatory pannus extended into the cornea, and polymorphonuclear leukocytes infiltrated far into the central area. The anterior chamber was filled with serous exudate and many clumps of monocytes and polymorphonuclear leukocytes were adherent to the corneal endothelium. The thickened iris was heavily and diffusely infilterated by plasma cells, especially in the deeper stromal layers.

The ciliary processes revealed similar changes and large numbers of plasma cells were being discharged into the posterior chamber, onto the zonule, and into the vitreous. There were several very dense accumulations of large mononuclears and polymorphonuclears within the vitreous. Some of these were partially surrounded by proliferating fibroblasts.

The preretinal vessels which were cuffed by dense accumulations of large and small mononuclears also contained similar cells within their lumens. They also exhibited adventitial proliferation of fibroblasts. The reaction was most intense in front of the nervehead where many mature plasma cells and occasional polymorphonuclears were also

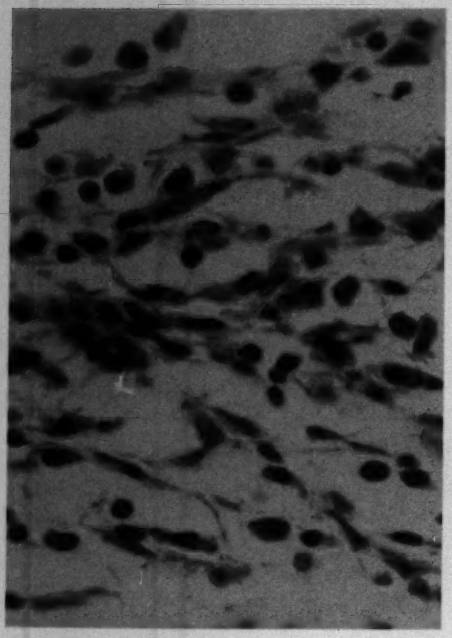

Fig. 12 (Zimmerman and Silverstein). From the same field shown in Figure 11-b magnified to ×630. The mononuclear infiltrate includes lymphocytes, monocytes, plasma cells in various stages of differentiation, and fibroblasts.

observed. These same cell types infiltrated the nervehead, leptomeninges, and choroid immediately adjacent to the disc.

A thin serous exudate was present beneath the recently detached retina posteriorly but there was no significant degeneration of the visual cells.

Twenty-three days. There was a very light infiltrate of lymphocytes and plasma cells at the limbus but the intraocular tissues were free of inflammatory reaction. The retina was in place but adjacent to the disc there were degenerative changes in the visual cells.

Thirty days. The limbal and anterior uveal tissues were lightly infiltrated by lymphocytes and plasma cells. A more marked mononuclear reaction was present posteriorly along the retinal vessels and on the surface of the optic disc. Exudation of large and small mononuclears and proliferation of fibroblasts were also observed in the posterior vitreous. Mild degenerative changes in the visual cells and a small focal buckling of the outer retinal layers were present immediately adjacent to the disc.

Discussion

Foss[1] and others[2-5] have postulated that the "primary uveitis" which appears spontaneously six to 14 days after a single intravitreal injection of antigen in rabbits is an anaphylactic reaction due to the presence of residual antigen within the eye and its interaction with specific antibody. According to Foss,[1] there is a constant incubation period, the duration of which is dependent on the specific qualities of the antigen employed, but not upon its concentration.

Our own observations are not in complete agreement, for by lowering the dose of antigen injected into the vitreous the incubation period is lengthened, the intensity and duration of the clinical reaction lessened, and the percentage of animals reacting reduced. In spite of the lack of clinical response to small doses (0.05-0.10 mg.) of crystalline egg albumin injected into the vitreous, the eye does become sensitized and will respond to a second challenging dose of antigen administered intravenously or intravitreally.[4] Furthermore, we have been able to confirm the observation made by Schlaegel and Davis[5] that there is histopathologic evidence of a late inflammatory response even in those rabbits which do not exhibit clinical manifestations of "primary anaphylactic uveitis."

The histopathologic changes observed after intravitreal injection of a single 1.0 mg. dose of crystalline egg albumin, during the first few days, consist of a mild, nonspecific acute inflammatory reaction followed by subsidence and relative quiescence. At about the fifth day, just before the onset of a clinically impressive uveitis, the limbal tissues begin to exhibit increasing infiltration by mononuclear inflammatory cells. Polymorphonuclear leukocytes and capillaries extend into the peripheral cornea. The iris, vitreous, and

preretinal tissues also begin to be infiltrated by mononuclear cells.

At first these mononuclear cells appear to be lymphocytes, monocytes, and undifferentiated mesenchymal cells. During the next few days they increase greatly in number and in cytologic variation. Some become macrophagic and contain particles of phagocytosed debris in their cytoplasm. Others develop elongated processes and appear to be differentiating as fibroblasts. Still others acquire an abundant basophilic cytoplasm, an eccentrically situated nucleus, and a paranuclear pale-staining cytoplasmic halo characteristic of plasma cells. Such plasma cell differentiation is most obvious at the limbus, in the deeper layers of the iris stroma, along the epithelial surface of the ciliary processes, in the immediate vicinity of the preretinal capillaries, and in the mesenchymal tissue in front of the optic disc.

The inflammatory reaction reaches its peak during the second week after injection and gradually subsides thereafter. During the height of the reaction in some of the eyes, agglutinated masses of large mononuclear cells form in the vitreous, on the inner surface of the retina and on the pupillary margin. Some resemble the "mutton-fat keratic precipitates" observed in granulomatous uveitis of man while others which are larger appear to be developing into tuberculoid granulomas. Also observed occasionally at this time are focal and diffuse round cell infiltrates in the choroid associated with serous exudation beneath the adjacent retina.

During the third week and thereafter, the inflammatory reaction subsides and most of the tissues gradually are returned to a normal microscopic appearance. Small collections of plasma cells in the limbus, iris root, over the ciliary processes, about the preretinal vessels, and on the nervehead are the last to disappear and can still be observed nine weeks after injection.

In addition to the reversible early changes resulting from increased vascular permeability, exudation and proliferation of inflammatory cells, there are secondary irreversible alterations in some eyes, particularly in the posterior segment. Exudative detachment of the retina with degeneration of visual cells may be seen in juxtaposition to the choroidal infiltrates. Later the formation of an inflammatory membrane along the inner surface of the retina leads to thickening and opacification of the retina. Contraction of this membrane produces fixed folds and permanent retinal detachment. Associated with these preretinal alterations are lesser degrees of intraretinal inflammation and gliosis. Gliosis of the nervehead may be marked. Thus even though the eye externally and anteriorly may appear to have returned to normal, the vitreous, retina, and optic nervehead may become irreversibly damaged. Such permanent changes, however, seem to occur only in those eyes which have exhibited a more severe clinical reaction.

The histopathologic changes observed after intravitreal injection of a single dose of 0.05 to 0.10 mg. crystalline egg albumin consist of an early, very mild nonspecific reaction to the injection. During the second week mononuclear cells begin to appear about the preretinal capillaries. By the beginning of the third week they are present in modest numbers in front of the disc, but the pronounced anterior segment reaction and exudation into the vitreous observed in the eyes which had received the 1.0 mg. dose is generally lacking. There were two exceptions to this generalization in the low-dose group. The rabbit killed on the 20th day had demonstrated a mild, late clinical response. It showed microscopically an exudative reaction anteriorly as well as posteriorly. The rabbit killed on the 30th day exhibited only very minimal arterior segment changes but posteriorly there was a moderate exudative and proliferative reaction along the retinal vessels and in the cortical vitreous.

In comparing the microscopic alterations after small doses of antigen with those observed after 1.0 mg. we note differences in time of onset, intensity of reaction, and site

of activity. With the small dose, the incubation period is lengthened, the mononuclear cell infiltrates appearing only after the first week. The reaction is typically very mild and confined to the preretinal and nervehead capillaries (figs. 11, 12, and 13). Most striking is the negligible uveal involvement (figs. 3 and 14), the absence of exudation into the ocular chambers, and the lesser degree of corneal reaction. Significantly, in both the large and small dose groups the reaction is principally mononuclear and with time a large proportion of the cells present typical cytologic features of mature plasma cells. The cornea, however, is the only tissue in which polymorphonuclear cells are conspicuous.

It is of some interest to speculate on the immunologic mechanisms responsible for this "primary anaphylactic uveitis" as it has been designated by Foss.[1] This name implicates an immediate type of hypersensitivity reaction dependent upon circulating antibody. Some of our own earlier studies summarized elsewhere[3,4] furnished additional evidence that both a sufficiently high titer of circulating antibody and a residual pool of unabsorbed antigen within the eye are required to support a clinically evident reaction. On the other hand, there is reason to conisder the participation of other immunologic mechanisms in the pathogenesis of this syndrome.

The sequence of events observed histologically in our studies would seem to be analagous to those observed by Gell and Hinde[6] during the early stages of active Arthus sensitization—that is, what they have termed "progressive immunization reaction."[7] These investigators injected rabbits intradermally with human globulin at intervals of two or three days, and followed the clinical and histopathologic changes which were produced by *each successive injection* into the same animal. The antigenic challenges were performed at varying stages

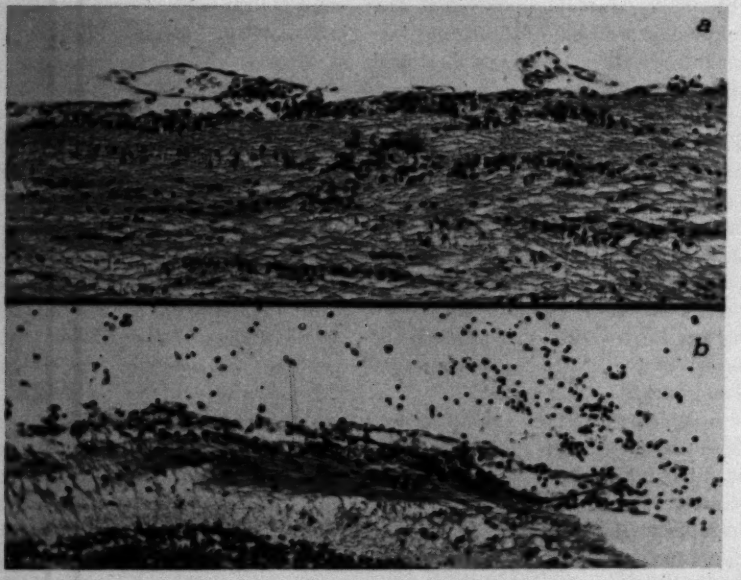

Fig. 13 (Zimmerman and Silverstein). (a) There is a very light perivascular infiltration of mononuclear cells as compared with the more pronounced exudation illustrated in (b). (a) is from rabbit 15P, eight days after intravitreal injection of 0.10 mg. crystalline egg albumin. (b) is from rabbit 75L, eight days after the 1.0 mg. dose. (Hematoxylin-eosin, ×165.)

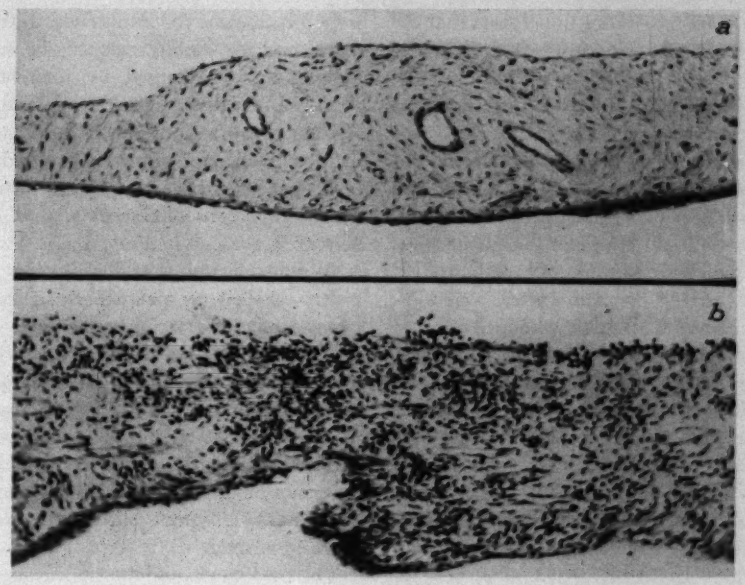

Fig. 14 (Zimerman and Silverstein). These two fields are from the same pair of rabbits as in Figure 13. The iris in (a) (after the 0.10 mg. dose) shows no significant infiltration while that in (b) (after the 1.0 mg. dose) is markedly infiltrated by mononuclear cells. (Hematoxylin-eosin, ×115.)

during the development of immunologic response, from the most mild early response to the later full-blown Arthus reaction.

Gell and Hinde found "that the earlier reactions are not, as might have been expected, very mild Arthus reactions, but consist entirely of mononuclear cells with the characteristic perivascular distribution. The later lesions, however, become complicated by the appearance, in the middle of the reacting zone, of a mild exudative polymorphonuclear reaction accompanied by vascular damage, which in turn increases in severity and extends over a wider area with each subsequent injection, until the lesions show all the features of the acute twenty-four hour Arthus reaction as usually described."[7]

They interpret the mononuclear cell response to the early injections of antigen as a manifestation of a delayed hypersensitivity component, appearing earlier than the strong, antibody-mediated Arthus reaction, and thus uncomplicated by the superposition of a polymorphonuclear exudate. When, after further injections, a good circulating antibody titer has appeared, the typical acute Arthus lesion is seen superimposed on and often masking this mononuclear response. They assume, then, that both the antibody-mediated hypersensitivity and a delayed or "tuberculin-type" hypersensitivity contribute to the ultimate Arthus lesions. Following the acute Arthus reaction "there is wholesale differentiation to cells of the plasma cell series, until by seven to fourteen days the foci consist largely of mature plasma cells; in the 'tuberculin-reaction,' although some differentiation in the same direction occurs, it is not nearly so widespread nor complete."[7]

In our studies, the injection of antigen into the vitreous has resulted in the formation of a depot, from which the antigen leaks out only slowly over a period of some weeks.[1,4] During this period, the rabbit is slowly being immunized, so that at a time when sensitivity is sufficiently well developed to support an inflammatory response, antigen which can effect this challenge is still resident

in the eye. The possibility thus exists that the conditions of our experimental system are in effect those of a continuously stimulated progressive immunity reaction, so long as antigen survives in the vitreous.

The inflammatory lesions which we observed within the uvea, retina, vitreous, and ocular chambers were not unlike those described by Gell and Hinde in the early stages of their developing immunity response. It was characterized by mononuclear cell infiltrates, without a polymorphonuclear vasculonecrotic Arthus component. However, the "perivascular-island" distribution of these cells was conspicuous only about the optic disc and retina; in the limbus, iris, ciliary body and choroid, there was a more generally diffuse distribution.

The vasculonecrotic component described by Gell[7] was not observed at any stage in our studies. This is, in all probability, best ascribed to the relationship existing between the rate of appearance of circulating antibody on the one hand and of disappearance of antigen from the vitreous on the other hand.

It has been shown that the dose-dependence of the Arthus response is such that appreciable amounts of both antigen and antibody must meet in order to incite a strong polymorphonuclear exudative response.[8] After a single intravitreal injection of antigen, the slow rise in antibody titer probably is insufficient to support a good Arthus response at a time when sufficient antigen remains in the eye. Conversely, when antibody finally appears in adequate amounts, the antigen remaining at this time has probably been reduced to a level too low to support a typical Arthus reaction. This is borne out by the observation[9] that a second intravitreal injection of antigen at this time will lead to a typical polymorphonuclear response.

The cornea was the only tissue in which polymorphonuclear leukocytes were conspicuous in the response to a single intravitreal injection of antigen. This infiltration is, however, of questionable significance for we[9,10] have observed an intense, diffuse polymorphonuclear infiltration of the cornea in guinea pigs challenged by intravitreal injection of antigen after active and passive Arthus sensitization, sensitization to tuberculin, and also in guinea pigs rendered delayed-hypersensitive to protein antigens by the method of Uhr, Salvin and Pappenheimer.[11]

In considering the immunologic mechanisms which may participate in the pathogenesis of these lesions, we must conclude that at this time no one mechanism finds unequivocal support from all directions. In considering the temporal factors involved, it is obvious that the long incubation period after injection of antigen cannot contribute information in favor of either "immediate" or "delayed" hypersensitivity as the mechanism involved.

On the one hand, the appearance of circulating antibody at the time that inflammation begins in the 1.0-mg. animals, and its absence or presence in only low titers in the clinically non-reactive low dose animals suggest a mechanism involving interaction of antigen with antibody.[4]

On the other hand, the histopathologic picture which we see is similar to that which Gell and Hinde[6] attribute to a delayed hypersensitivity mechanism. They point out that the antibody-mediated immediate wheal and erythema response is one consisting primarily of edema, while the antibody-mediated Arthus reaction is characterized by a predominantly polymorphonuclear response.

We may conclude that the inflammatory response in the rabbit eye to a single injection of crystalline protein into the vitreous is in all probability due to a complex of immunologic events. In addition to the late stage of local antibody production which is manifested by the plasma cell differentiation, these may include the wheal and erythema response, perhaps a modified type of Arthus response, and additionally a contribution by a delayed-hypersensitivity mechanism. The latter component must always be considered

in any lesion produced in an actively sensitized animal, although the extent of its participation in the present instance is unclear.

Two additional points must also be made: (1) that traditionally the basic immunological mechanisms have been defined in the experimental animal in terms of the acute inflammatory response to a single challenge with antigen, rather than as slowly developing responses more typical of natural disease processes, such as we are dealing with in the present study; (2) that the anatomy and physiology of the ocular tissues may be such as to modify both the temporal and the histopathologic development of an immunogenic inflammatory response.

Summary

1. Histopathologic observations have been made on rabbit eyes given single intravitreal injections of crystalline egg albumin. One series received 1.0 mg. ("large-dose") which produced a clinically apparent uveitis in all injected eyes after a latent period of six to eight days. The other series received 0.05 or 0.10 mg. ("small-dose") which, with one exception, failed to provoke a clinically significant inflammatory reaction.

2. In both the large and small dose series, the intraocular reaction was principally mononuclear, but in the eyes given the small dose there was virtually no uveal reaction while those given the large dose exhibited pronounced iridocyclitis and chorioretinitis.

3. Although in most cases the inflammatory reaction was entirely nongranulomatous, a few eyes at the height of reactivity exhibited granulomatous lesions.

4. Complications were not observed in the small-dose series but in some of the eyes injected with the large dose the following were noted: retinal detachment, degeneration of visual cells, preretinal membrane-formation, fixed folds of retina, degeneration of vitreous, and gliosis of retina and optic disc.

5. The spontaneous inflammatory response observed in these eyes has been compared to the "progressive immunity reaction" described by Gell and Hinde.[6,7] These lesions have been discussed in terms of the fundamental immunological mechanisms which might contribute to their pathogenesis.

Armed Forces Institute of Pathology (25).

Acknowledgment

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Discussion

Dr. Seymour P. Halbert (New York): A comparatively short time ago immune hypersensitivity seemed like a relatively simple phenomenon. There has been a rapid expansion of knowledge in this field in recent years, and the basic mechanisms now appear more and more complex.

All hypersensitive or allergic reactions are rather specific and apparently *all* require previous contact with the inciting agent. Manifestations may be quite variable depending upon the tissue involved, the dosage, route, species, allergin, and so on. In general, however, there are two classes of hypersensitive response—the immediate, such as wheal and erythema reaction, hay fever, the Arthus reaction, anaphylaxis; and the delayed type of response, for example, infectious allergies in tuberculosis or streptococcal disease; contact dermatitis, and so on.

These general classes of hypersensitivity differ in several ways. The immediate reaction has a shorter incubation period. The immediate reaction can be transferred by serum from a sensitive individual to a nonsensitive individual. The delayed type of reaction cannot. It cannot be transferred by serum, but it can be transferred by certain cells of the sensitive individual.

In addition, if one adds antigens in vitro to cells from an individual with the immediate type of allergy, no damage occurs; but with delayed hypersensitive individuals the antigen is cytotoxic for the cells from the sensitive individual in vitro.

We now realize that many hypersensitive reactions are not so clear-cut as this—that probably a number of the fundamental mechanisms act in the development of tissue lesions, and that many lesions are in all likelihood composite in nature. The authors are to be highly praised for their concerted attack on this problem as related to the eye, and for the application of quantitative experiments in attempts to unravel the sequence of events involved in the reactions.

In their studies with a single antigen dose, a very basic question has been raised by their findings: How much of the cellular response was due to the damaging effect of antigen-antibody reaction, that is, hypersensitivity; and how much was due to the simple marshaling of antibody-forming cells to the site of deposition of antigen? Information on this point could conceivably be obtained by the use of fluorescent antibody tagging methods, in which one can detect the presence of antibody-forming cells with suitably fluorescein-labeled materials. Should a very large proportion of the round cells that they find in some of their tissue lesions be shown in this way to be synthesizing antibody at the peak of the tissue reaction, this would suggest that in this system, at least, much of the cellular reaction would be due to the simple process of marshaling and bringing forth the antibody-forming cells to the site.

In connection with this, it would be of interest to see how much of the cellular response might be due to the mere process of removing comparatively large molecular weight substances from the vitreous in the absence of antibody formation. This question might be answered by the use of non-antigenic large molecular weight material such as gelatin; or some nonantigenic polysaccharides such as certain of the pneumococcal polysaccharides which are nonantigenic for the rabbit.

Recently Germuth has reported some very intriguing demonstrations of hypersensitive antigen-antibody reactions in the avascular cornea, as Dr. Maumenee has mentioned. I would be curious to know if there is histologic data available on the cellular sequence of this reaction as compared to the ones found in the vitreous by Dr. Silverstein.

In another connection, last year at the San Francisco meeting, Dr. Maurice of London presented data on the diffusion of protein out of the rabbit vitreous. His experiments indicated that approximately 16 percent of the amount present at any time escapes per day—a rather slow rate of absorption—and his studies indicated that most of the absorption was by simple diffusion, mostly toward the anterior chamber. There is evidence that certain adjuvants which boost the antibody response, act to a large extent by slowing the rate of absorption of antigens. I wonder, therefore, whether the antibody response in rabbits given 1.0 mg. of egg albumin into the vitreous is significantly higher than the antibody response of the same dose given to rabbits intravenously.

Lastly, and perhaps most importantly, in relation to human disease, is it possible to produce ocular hypersensitive reactions with solely extraocular administrations of antigens?

Dr. A. E. Maumenee: What was the time interval between the systemic administration of the antigen and the focal reaction in the eye? Usually the focal reaction in a clinical case occurs 24 to 48 hours after the systemic injection of an antigen. Did it take that long to develop a reaction in the eye of the rabbit?

Dr. Michael I. Wolkowicz (Philadelphia): I would like to ask Dr. Silverstein if he found any ciliary response in the control in the second eye. The control eye usually will show the presence of antibody, just like the eye which is originally inoculated.

Dr. Arthur M. Silverstein (closing): Perhaps I had better take the questions backward, because I think I can do more justice to the last questions than the first one which Dr. Halbert brought up.

With respect to the second eye: In the instances in which we obtained the spontaneous response to the injection of antigens—spontaneous response a week or eight days later—usually (if not always) we could find no reaction in the second, untreated eye—nothing that we thought deviated significantly from normal.

With respect to how long it takes for the focal reaction Dr. Maumenee asked about: When we give antigen intravenously, some weeks after the intra-

ocular antigen, we can see the eye react almost immediately. Sometimes we see contraction of the pupil. Certainly within 30 minutes we can see the injection of the vessels at the limbus and the vessels in the iris. The reaction does not reach a peak, however, until about 24 hours; but the reaction starts almost immediately.

This again brings up the last question. When we get a very marked reaction in response to intravenous antigen, occasionally in the second eye, we see a very transient and very mild inflammatory reaction. This is in the untreated eye. The treated eye reacts very violently. The untreated eye usually will not react; but occasionally, as has been reported by others, it will react very mildly. Why this should be, I don't know.

With respect to the very provocative questions that Dr. Halbert asked, and to his suggestions; they are so numerous that there is neither time nor wit enough on my part to satisfy them all. I can discuss one or two of them, however.

Some years ago Dr. Witmer and Dr. Goldmann applied the fluorescent antibody technique to the study of antibody production in the eye, and showed indeed that quite a number of the round cells which they found in those eyes were producing antibody. I think perhaps, in extension of what they found, that one would like to know, at the later stages and with a lesser amount of antigen than they used, what the results would be. We are starting such studies now, as well as other studies, some of which Dr. Halbert suggested; perhaps we will be able to discuss them more intelligently at next year's meeting.

EXPERIMENTAL ASPECTS OF OCULAR SIDEROSIS AND HEMOSIDEROSIS*

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INTRODUCTION

In a previous study,[1] evidence was presented that dogs subjected to repeated intravenous injections of colloidal iron† or repeated transfusions of compatible blood gradually develop degenerative changes in the pigment epithelium and the retina. These changes present many of the histopathologic features of siderotic retinopathy and also resemble retinitis pigmentosa.

The present study deals with experimental aspects of ocular siderosis and hemosiderosis induced in rabbits, dogs, and cats by injections of diluted saccharated iron oxide (SIO) or of autogenous blood into the anterior chamber, the vitreous body, or systemic circulation of the living animal. The in vivo experiments were supplemented by analogous experiments dealing with SIO-injections into the enucleated eyes of man and monkey.

The use of SIO as of many other commercially available protein-free colloidal iron preparations offers the following advantages: (1) side-effects resulting from protein breakdown products are avoided: (2) the colloidal dispersion of iron in aqueous solution remains stable over a wide pH range; (3) the iron complex does not precipitate in blood or serum; (4) the iron dispersion in aqueous humor and vitreous body occurs relatively rapidly unless it becomes bound to mucoid matter or other siderophilic substances; (5) its interaction with extracellular siderophilic tissue components can readily be demonstrated histochemically (Prussian blue reaction for the trivalent, Turnbull's blue reaction for the bivalent form of iron); (6) the physiococochemical structure of the inorganic ferric hydroxide molecules can be differentiated from the organic aggregates of the iron complex in ferritin* and hemosiderin by electron micros-

* From the Department of Ophthalmology and the Oscar Johnson Institute, Washington University, School of Medicine. This research was supported in part by a grant (No. B-1789) from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service.

† Ferrivenin (Benger's Ltd.), Proferrin (Merck, Sharp, and Dohme). The latter was used in this study. It contains 20 mgm of elemental iron per cc. at a pH of 10.8. In the experiments with intraocular injections, dilutions with physiological saline in a ratio of 1:10 were employed.

* Ferritin is formed in the cytoplasm of iron ingesting cells by the combination of trivalent iron with apoferritin (α_2-globulin) (Farrant,[2] Bessis and Breton-Gorius,[3,4] Richter[5,6]).

copy (Richter[3]). Further differentiation is based on the failure of ferrintin to give a positive Prussian blue reaction, whereas SIO and hemosiderin stain blue.

Method

I. In vivo injections of SIO or blood

A. Anterior chamber

1. In this part of the study, twenty healthy adult albino rabbits were used. About 0.1 cc. of SIO diluted with physiological saline in a ratio of 1:10 (0.2 mg. of elemental iron) or 0.1 cc. of undiluted autogenous blood was injected at the limbus into the anterior chamber of the right eye. Equal amounts of physiological saline were injected into the left eye (control). A No. 26 gauge needle was employed. In either eye, an equivalent amount of aqueous humor had been released prior to the injection.

2. The eyes were enucleated under Nembutal anesthesia at a given time following the injections varying from 15 minutes to one year.

3. Immediately following the enucleation, the the eyeballs were fixed in formalin for the histologic work-up.

4. Cross section were stained with Heidenhain's hematoxylin-eosin, Gomori's stain for the demonstration of trivalent iron, and van Gieson's staining technique.

B. Vitreous body

1. Injections of 0.1 to 1.0 cc. of SIO diluted with physiological saline in a ratio of 1:10 (0.2 to 2.0 mg. elemental iron) were injected into the vitreous body of the right eye of 30 albino rabbits, four dogs, and twelve cats. A No. 26 gauge needle was used. The site of the injection was either the equator (42 animals) or the center of the cornea. In the former case, a small amount of vitreous body was released prior to the injection. In any case, the attempt was made to maintain an intraocular pressure of less than 35 mm. Hg during the injection. In the case of the transcorneal injection, the needle was led through the lens under rotatory motion as employed in trephine operations until the tip of the needle reached the middle of the eyeball. Only 0.1 cc of the SIO solution or the blood was injected in those cases in order to avoid undue rise of the intraocular pressure.

2. The eyes were enucleated at intervals varying from half an hour to one year following the injection.

3. Flat preparations of the retina were prepared as follows:

The eyeball was dissected at the equator into an anterior and posterior half. The posterior segment was cleaned of the vitreous body and the specimen rinsed with 0.9-percent saline solution to wash out the excess of the injected agent. Thereafter, the specimen was immersed for 10 minutes into a four-percent formaldehyde solution and then rinsed again with tap water for a few minutes. This was followed by immersion of the specimen into a mixture of equal parts of 10 percent of hydrochloric acid and potassium ferrocyanide, where it remained for about 10 minutes. After washing with tap water the specimen was immersed into a 70-percent alcohol solution for 10 minutes. Finally, the retina was loosened and detached from the underlying layers and its insertion at the optic nerve severed. The detached retina was then spread out on a glass slide and passed through increasing concentrations of alcohol and finally dehydrated by passing through xylene prior to embedding of the flat preparations in Gum Damar.

C. Systemic circulation

1. In this part of the study 24 rabbits and six dogs were employed. They were subjected to either single or repeated injections of SIO (2.0 to 20 mg. elemental iron/kg. body weight).

2. The eyes were enucleated at intervals varying from 15 minutes to two years following the last injection.

3. Flat preparations of the retina, the ciliary body and iris as well as cross sections of duplicate eyes were prepared as outlined above.

II. Postmortem experiments

Six freshly enucleated monkey eyes and six human eyes obtained at autopsies were used for perfusion of the anterior chamber with a diluted SIO-solution (containing 2.0 mg. elemental iron per cc.) for a period of one hour at a pressure of 35 mm. Hg. An equal number of eyes were employed for staining of the retina by injection of SIO into the vitreous body.[13] The flat preparations and cross sections obtained from these eyes were worked up the same way as in the vivo experiments. In addition, flat preparations of the cornea-scleral segments from which the iris and ciliary body have been removed were prepared. They were mounted on glass slides with the outer surface pointing towards the cover slip.

Results

The results of the in vivo and postmortem experiments with SIO-injections into the anterior chamber revealed that the SIO molecules are able to leave the eye via the trabecular meshwork, and the outflow channels of Schlemm's canal within minutes. However, large quantities of SIO were retained in the trabecular meshwork of all animals.

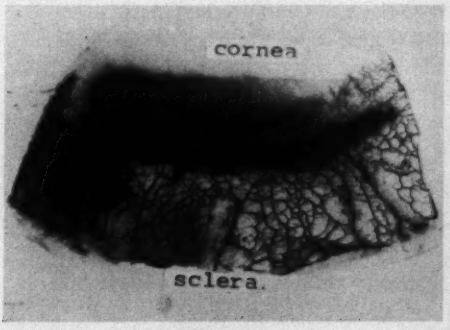

Fig. 1 (Cibis and Yamashita). Flat preparation of limbal and postlimbal area of a normal monkey eye depicting positive Prussian blue stain of the trabecular meshwork and of the outflow channels of Schlemm's canal after perfusion of the anterior chamber of the freshly enucleated eye with diluted saccharated iron oxide (SIO) (containing 2.0 mg. of elemental iron per cc.) for one hour at about 35 mm. Hg pressure.

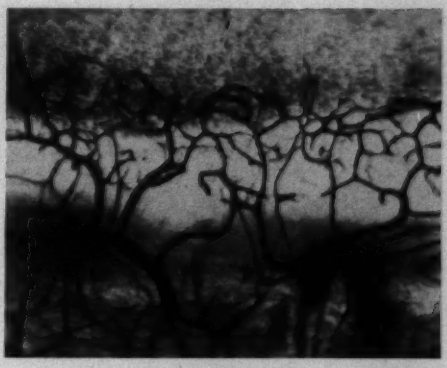

Fig. 2 (Cibis and Yamashita). The same after removal of the scleral trabecula and of Schlemm's canal by dissection with a razor blade. Notice free ends of outflow channels.

In the eyes of man and monkey, the endothelial cell of Schlemm's canal and of the outflow channels as well as the perivascular tissues elicited a marked Prussian blue reaction. This could be demonstrated particularly well in full thickness flat preparations of the anterior segment, from which the ciliary body and iris were removed (figs. 1 to 4). Hyaluronidase (Wydase) somewhat impaired but did not prevent the iron uptake.

It is generally assumed that the bulk of normal perivascular tissue consists of acid mucopolysaccharides (AMP). The vitreous body is particularly rich in hyaluronic acid (Meyer[10]). Injections of SIO into the vitreous body led to large uptake of iron by the vitreous and by the perivascular tissues. In rabbits, the perivascular tissue of the retina (figs. 5 and 6) as well as of the optic nerve (fig. 7) gave a positive Prussian blue reaction in less than 30 minutes following the injection of the SIO. An example of iron staining of perivascular tissue in a human retina is shown in Figure 8. Similar observations have been made previously by Weed and Wegefarth,[11] and Berens and Posner[12] in cross sections of rabbits and human eyes.

Linkage of iron to siderophilic components in the vitreous body and the perivascular tissue of the retina may persist for many months (figs. 9 and 10). This is asso-

Fig. 3 (Cibis and Yamashita). Flat preparation of limbal and postlimbal area of a human eye perfused with SIO (2.0 mg. Fe/cc) for one hour, four hours after death. Notice heavy Prussian blue reaction of the trabecular meshwork, Schlemm's canal, and perisvascular tissue.

ciated with a gradual disintegration of the affected tissue structures, fibrotic changes, and eventually sclerosis and obliteration of the retinal blood vessels. Degeneration of the neural structures in the retina takes place concurrently.

Following intravitreal injections of SIO or autogenous blood (table 1), rabbits developed retinitis proliferans after two weeks. The proliferation of fibrous tissue originated either from the prepapillary tissue (fig. 11) or by ingrowth of fibrocytes from the site of injection (fig. 12). Examples of capillary proliferation at the inner surface of the retina are shown in Figures 13 and 14. Detachment of the hyaloid and of the internal limiting membranes resulted in some cases from massive vitreous retraction (fig. 15). Some signs of degeneration of the retina and gliosis were observed after more than two weeks in almost all cases treated with SIO or blood. This occurred not only in connection with retinal detachment but also related to hemosiderin or siderin deposition on the inner surface of the retina with or without stainable iron molecules in the neural tissue (fig. 16). In four cases, the injection of SIO was administered through

Fig. 4 (Cibis and Yamashita). Enlarged picture of episcleral veins depicted in Figure 3. Note heavy iron stain of the perivascular tissue.

TABLE 1

RESULTS OF INTRAVITREAL INJECTIONS

Type of Pathological Manifestations	Ratio of Animals Affected to Number of Animals Treated	
	(a) With SIO*	(b) With Blood†
1. Retinitis proliferans	16/20	6/8
2. Vascular proliferation	1/20	3/20
3. Choroidopathy, cyclopathy, and iridopathy	3/20	—

* 0.2 to 2.0 mg. elemental iron.
† 0.1 to 1.0 cc. autogenous blood.

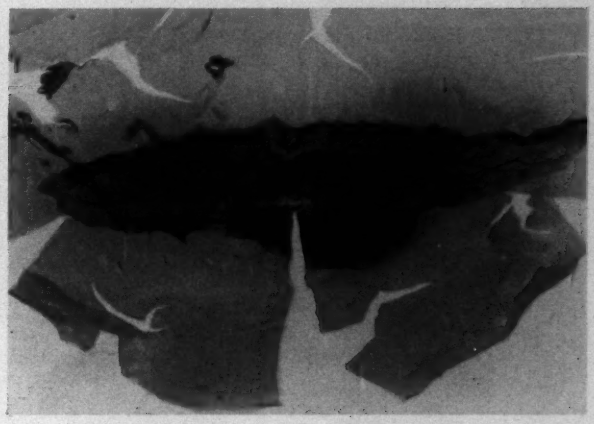

Fig. 5 (Cibis and Yamashita). Flat preparation of rabbit retina depicting vascular pattern over the medullated wing after injection of diluted SIO (0.2 mg. Fe) into the vitreous body, one hour prior to enucleation. Prussian blue reaction elicited by exposure of the excised retina to Pearl's reagents.

the cornea and lens. Nevertheless, degeneration of the retina occurred and was associated with fibrous tissue and vascular proliferation at the inner surface of the retina. None of these changes were observed in control eyes in which saline had been used instead of SIO or blood.

Secondary degeneration of the pigment epithelium generally accompanied severe retinal changes. Primary degeneration of most severe form in the pigment epithelium was observed whenever the injected solutions had entered the subretinal space through a retinal break (fig. 17). A detailed description of the experiments and results related to retinitis proliferans and vascular proliferations induced by iron or blood will be presented elsewhere.[14]

The presence of nonprecipitate iron in the cytoplasm of endothelial cells of the cornea, of the trabecular meshwork, and of Schlemm's canal, a few hours after the injection of SIO into the anterior chamber, is depicted in Figures 18 to 20. Experimental and clinical evidence[15, 16] seems to indicate that the uptake of iron by endothelial cells is reversible as long as the conversion into siderin or hemosiderin has not taken place. Severe and continuous iron overload of the cytoplasm is said to lead to an exhaustion of

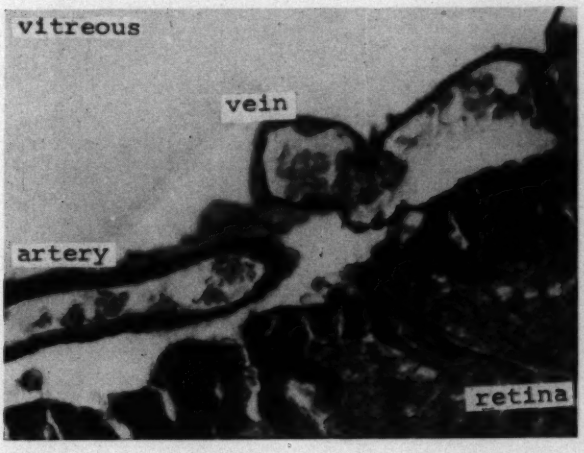

Fig. 6 (Cibis and Yamashita). Cross section of rabbit retina depicting positive Prussian blue reaction of the perivascular and prepapillary tissue after exposure to SIO (0.2 mg. Fe) for 15 minutes immediately following the enucleation. Note negative Prussian blue reaction of the endothelium and medial layer of the artery, positive reaction throughout the wall of the vein. (Yamashita and Cibis, Arch. Ophth., 61:698-708, 1959.)

Fig. 7 (Cibis and Yamashita). Longitudinal section of the optic nerve of an albino rabbit demonstrating positive Prussian blue reaction of the perivascular tissue and of adjacent septa and tributaries, four hours after the injection of SIO (0.2 mg. Fe) into the vitreous body of the living animal. Heavily stained parts of retina and adherent vitreous body at left. (Yamashita and Cibis, Arch. Ophth., **61**:698-708, 1959.)

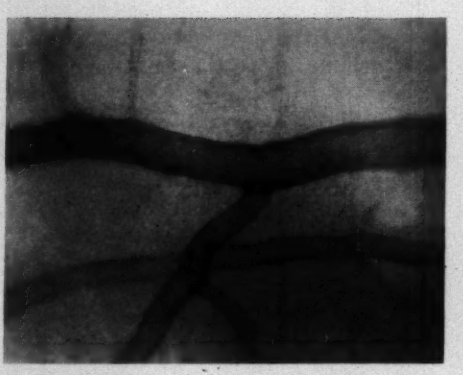

Fig. 8 (Cibis and Yamashita). Flat preparation of human retina exposed to SIO (20 mg. Fe/cc.) for four hours, five hours after death. Gomori's stain for iron.

Fig. 9 (Cibis and Yamashita). Flat preparation of rabbit retina with poor presentation of blood vessels due to degeneration of vascular branches four months after injection of SIO (2.0 mg. Fe) into the vitreous body of the living animal. Poor Prussian blue reaction of blood vessels which retained iron and did not obliterate completely. PAS stain failed to elicit other vessels.

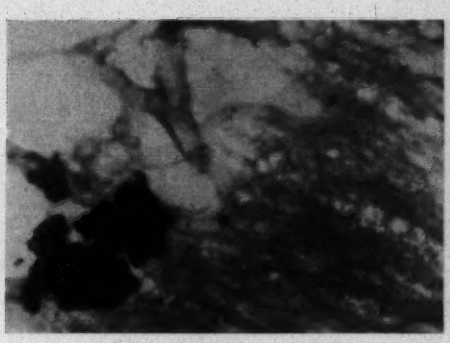

Fig. 10 (Cibis and Yamashita). Same as Figure 9. Cross section depicting iron containing walls of degenerated blood vessels. Heavy clumps of iron staining material in the retina close to faintly staining remnants of blood vessels.

apoferritin production; hence, siderin and hemosiderin become manifest in form of Prussian Blue positive granular material (fig. 20) and degeneration changes and cytolysis may follow. Almost complete denudation of the corneal epithelium may result from this condition. Similar observations were made with reference to the trabecular meshwork, the ciliary epithelium, and the epithelial layers of the ora serrata.[16] Cytoplasmic swelling seems to antecede the disintegration of the cells and may represent an essential factor in the mechanism of secondary glaucoma which so frequently accompanies anterior chamber hemorrhages (fig. 21). An enlarged picture of phagocytic cells in the trabecular meshwork is presented in Figure 22. Examples of extensive siderosis of the ciliary epithelium and of histiocytic elements of the inner surface of the retina and the vitreous body are depicted in Figures 23 and 24.

The effect of systemic siderosis and hemosiderosis on the eye is of considerable interest. Single intravenous injections of SIO (2.0 to 20 mg. iron per kg. body weight) result in an excessive overload of the blood.

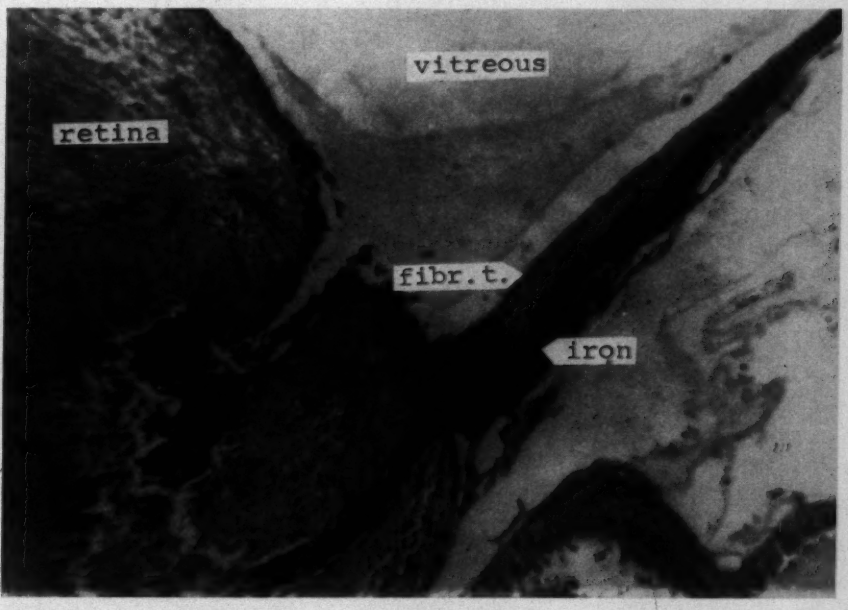

Fig. 11 (Cibis and Yamashita). Cross section of rabbit retina close to optic nerve head depicting proliferation of fibrous tissue to both sides of condensed vitreous with heavy uptake of iron, one month after the injection of SIO (0.2 mg. Fe) into the vitreous body of the living eye. Giemsa stain and Prussian blue reaction.

Fig. 12 (Cibis and Yamashita). Cross section of rabbit retina with ingrowth of fibrous tissue at site of transcleral injection of SIO (0.2 mg. Fe) into the vitreous body two months prior to death.

Fig. 13 (Cibis and Yamashita). Cross section of rabbit eye depicting total detachment of retina with fibrous tissue proliferation in the vitreous body and vascular proliferation on the inner surface of the retina, four weeks after injection of autogenous blood into the vitreous body. Condensed fibrous tissue close to the site of the scleral injection. Prussian blue and Giemsa's stain.

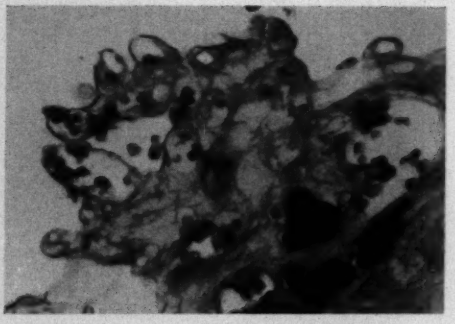

Fig. 14 (Cibis and Yamashita). Cross section of vascular proliferation with clumps of iron staining material in phagocytes, eight months after injection of SIO (0.2 mg. Fe into the vitreous body). Note absence of stain in neovascular tissue. Gomori's stain for iron.

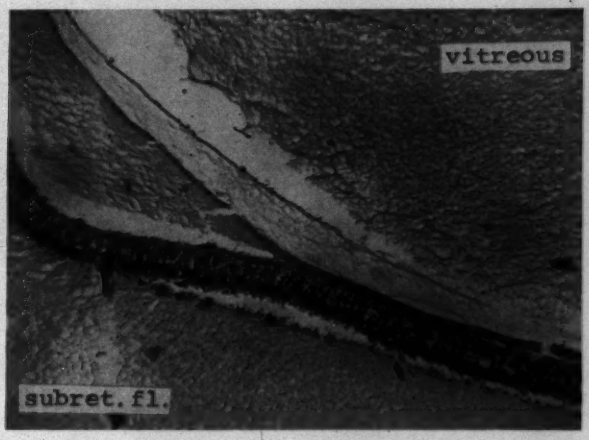

Fig. 15 (Cibis and Yamashita). Cross section of a detached rabbit retina with detachment of hyaloid and internal limiting membranes due to vitreous retraction four months after the vitreal injection of 0.1 cc. SIO (0.2 mg. Fe) Gomori's stain.

Uptake of iron in the cytoplasm of the endothelial cells of intraocular blood vessels can be demonstrated within 15 minutes. Diffusion of SIO into the stroma of the choroid and the ciliary body becomes manifest within two or three hours. In the ciliary stroma the SIO retains a nonprecipitate appearance. Thereafter, the Prussian Blue reaction in the stroma decreases and the accumulation of granular iron in the cytoplasm of phagocytic and endothelial cells in the ciliary processes, the choriocapillaris, the choroid and retina can be followed over weeks and months (figs. 25, 26, and 27). In dogs, siderotic granules in endothelial cells of ocular blood vessels and, in one instance, in Schlemm's canal could be demonstrated even seven years after the repeated injections of SIO or blood.[1] If the intravenous injections of SIO are repeated on several subsequent days, diffusion or SIO molecules into the iris stroma becomes noticeable after three or four days.

From histologic sections, in such cases, evidence can be elicited that the iron in the iris stroma is derived from blood vessels of ciliary processes which are attached to the posterior surface of the iris. The Prussian blue reaction is found generally restricted to the area immediately adjacent to the base of these processes and invariably exhibits a diffusion gradient indicative for such a

Fig. 16 (Cibis and Yamashita). Siderin deposition at inner surface and within rabbit retina, four weeks after injection of SIO (0.2 mg. Fe) into the vitreous body. Gomori's stain.

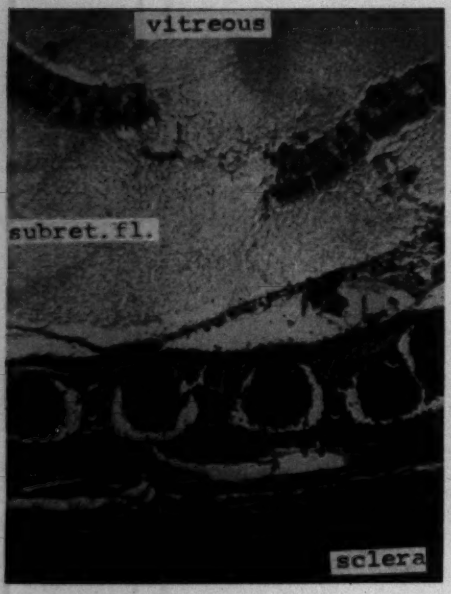

Fig. 17 (Cibis and Yamashita). Retinal detachment with a retinal break, degeneration of the pigment epithelium and severe congestion of the choroid about six months after injection of SIO (0.2 mg. Fe) into the vitreous body. Gomori's stain.

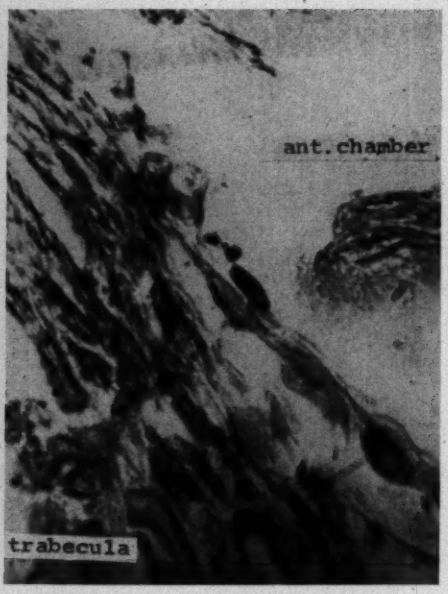

Fig. 19 (Cibis and Yamashita). Cytoplasmic iron uptake in endothelial cells of the trabecular meshwork, four hours after injection of SIO. Albino rabbit. Gomori's stain (0.2 mg. Fe).

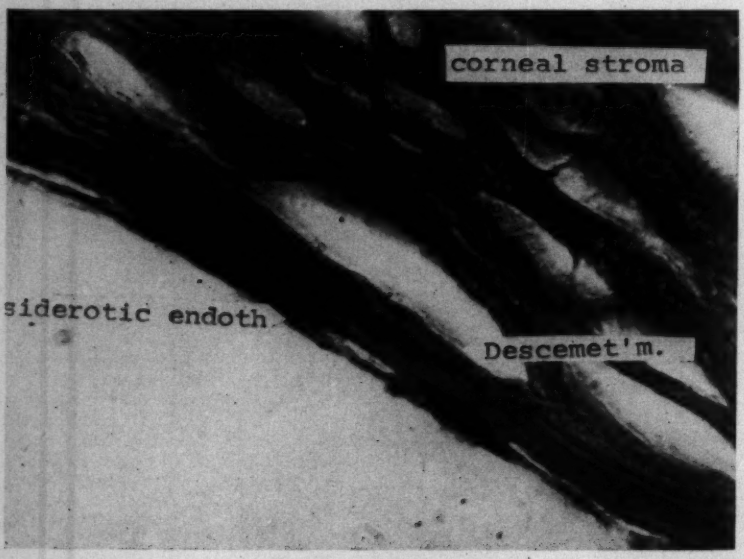

Fig. 18 (Cibis and Yamashita). Cytoplasmic uptake of iron in endothelial cells of the cornea, four hours after injection of 0.1 cc. of SIO (0.2 mg. Fe) into the anterior chamber of an albino rabbit. Gomori's stain.

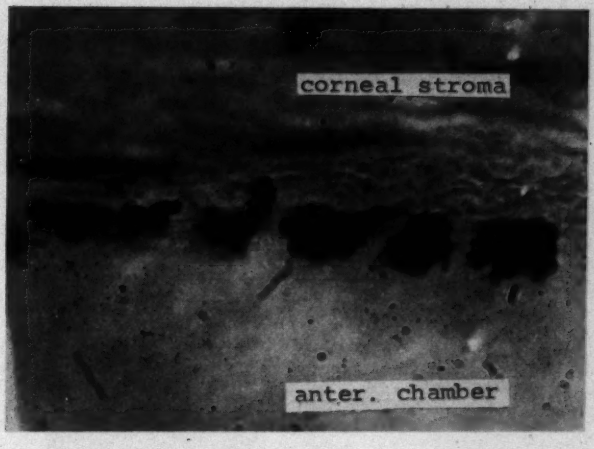

Fig. 20 (Cibis and Yamashita). Cytoplasmic swelling of corneal endothelial cells due to accumulation of granular Prussian blue positive material, two weeks after injection of SIO (0.2 mg. Fe). Albino rabbit. Gomori's stain.

Fig. 21 (Cibis and Yamashita). Cross section of anterior chamber of an albino rabbit one week after injection of SIO (0.2 mg. Fe) into the anterior chamber. Phagocytes in angular meshwork at the base of the ciliary body, at the surface of the iris and within the corneal stroma. Siderin in endothelial cells, note the greater number of phagocytes in the corneal stroma at the limbus where the endothelium and Descemet's membrane blend into the trabecular meshwork. Gomori's stain.

mechanism. That the iris tissue in itself possesses a great affinity to iron is illustrated by Figure 28, demonstrating that SIO is readily absorbed from the anterior chamber. In the instances in which the daily intravenous injection of SIO were continued over the period of a month, SIO molecules appeared in the endothelial cells of the trabecular meshwork and of Schlemm's canal, in the ciliary and the retinal pigment epithelium be-

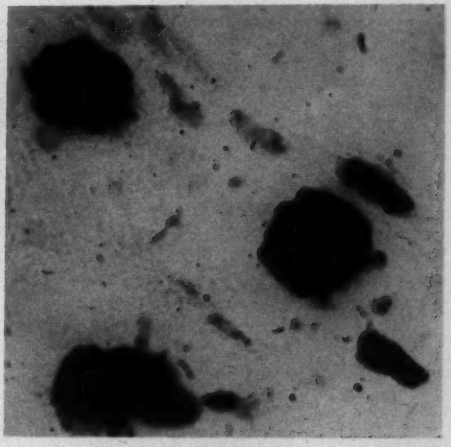

Fig. 22 (Cibis and Yamashita). Same as Figure 21. Enlarged picture of individual phagocytes depicting siderin aggregation in the cytoplasm.

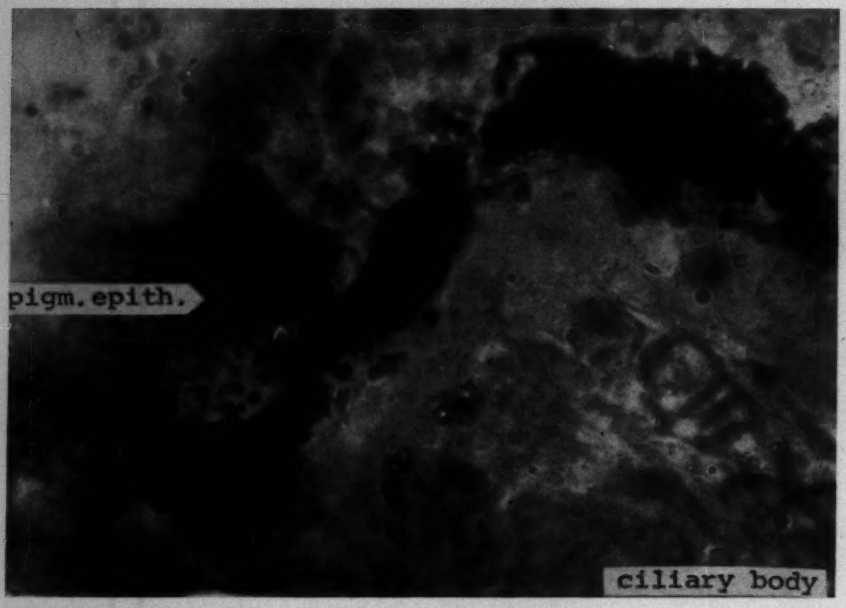

Fig. 23 (Cibis and Yamashita). Heavy cytoplasmic siderosis of a ciliary epithelial cells of a human eye which suffered from a long lasting intraocular hemorrhage. Gomori's stain.

side the endothelial cells of intraocular blood vessels. Even after seven years, residual siderin granules or transfusional hemosiderin could be detected in ocular structures.[1]

Comment

The experimental findings presented in connection with this study suggest at least two mechanisms by means of which patho-

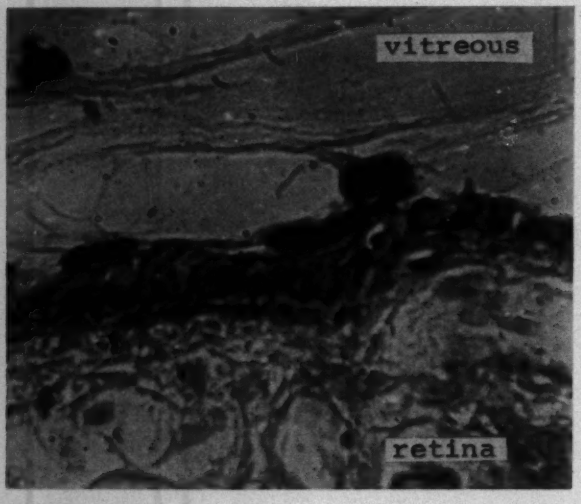

Fig. 24 (Cibis and Yamashita). Siderin granules in histiocytes of vitreous body and at the inner surface of the retina, one month after intravitreal injection of SIO (0.2 mg. Fe). Albino rabbit. Gomori's stain.

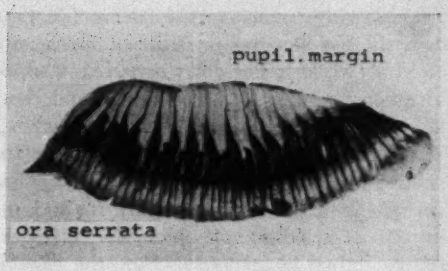

Fig. 25 (Cibis and Yamashita). Flat preparation of iris and ciliary processes of an albino rabbit stained for iron, one week after intravenous injection of SIO (20 mg. Fe/kg). Note positive Prussian blue reaction in ciliary processes, negative one in iris. (Yamashita and Cibis, Arch. Ophth., **61**:698-708, 1959.)

Fig. 26 (Cibis and Yamashita). Flat preparation of rabbit iris with ciliary processes on posterior surface stained for iron (Gomori), one hour after intravenous injection of SIO (20 mg. Fe/kg.). Positive iron stain of ciliary processes. Iris free of iron.

logic concentrations of iron in the eye may exert their deleterious effects upon the intraocular structures. The first one postulates the irreversible binding of iron to siderophilic substances, particularly the acid mucopolysaccharides (AMS) of the vitreous body, the perivascular tissue of the retina

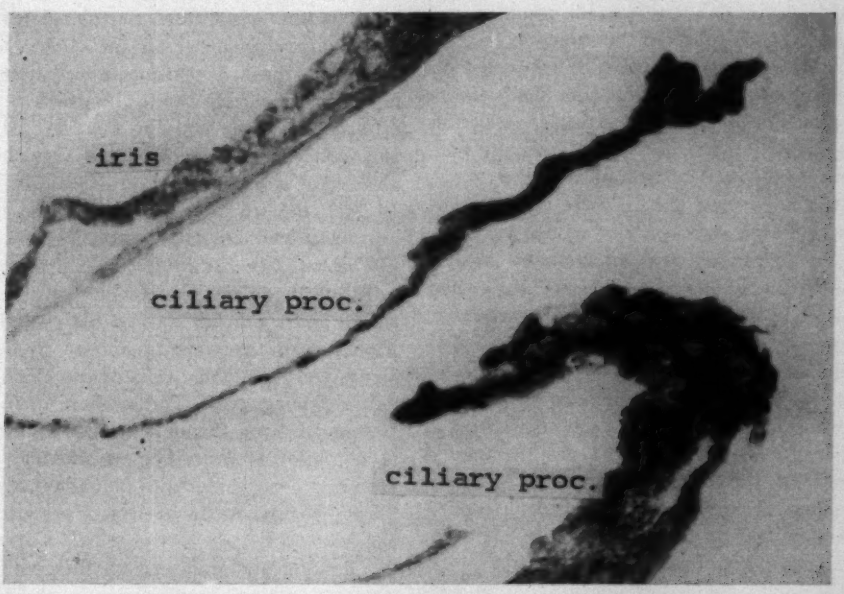

Fig. 27 (Cibis and Yamashita). Cross section of ciliary process and iris of an albino rabbit stained for iron, ten days after intravenous injection of SIO (20 mg. Fe/kg.). Positive Prussian blue reaction restricted to endothelial cells and phagocytes in ciliary body. Iris free of iron.

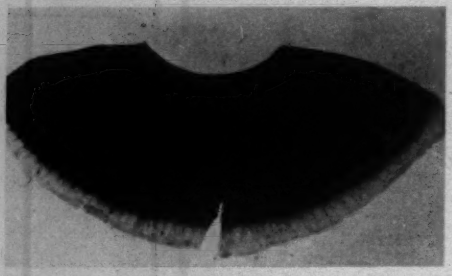

Fig. 28 (Cibis and Yamashita). Flat preparation of the iris of an albino rabbit showing heavy uptake of iron at the anterior surface, half an hour after SIO injection into the anterior chamber. The darkening of the iris tissue is caused by heavy positive Prussian reaction.

and optic nerve, the trabecular meshwork, and the perivascular tissue of the outflow channels of Schlemm's canal. A second proposed mechanism involves cytologic changes in the eye induced by the uptake of iron into the cytoplasm of endothelial and epithelial cells.

The sclerosis and obliteration of the retinal blood vessels as well as the proliferative changes which followed the injection of SIO into the vitreous body may be explained on the basis of a direct stimulation of fibroblastic elements by iron or the possible deletion of an inhibitory factor which normally prevents the growth of fibrous tissue.

It is interesting that in three or four rabbits kept alive over a period of one year following the intravitreal injection of saccharated iron oxide, the vitreous body had cleared to such a degree that the vascular changes in the retina could be followed ophthalmoscopically and photographed. In all four cases, the vascular obliteration and proliferative changes were associated with a marked demyelinization of the medullated wings of the rabbit retina. The question whether similar atrophic and proliferative changes can be induced experimentally in other species is under investigation.

Of pathogenetic significance is the finding that the degenerative changes following experimentally induced systemic siderosis developed with a much greater delay (years) than those following intravitreal injections of SIO (weeks). The delay occurred in spite of extensive uptake of iron by the endothelial cells of the intraocular blood vessels. This suggests that the linkage of iron to siderophilic matter in the perivascular tissue perhaps is of greater importance for the induction of sclerotic and obliterative changes of the retinal blood vessels than the effects of iron.

Sclerosis and obliteration of the outflow channels of Schlemm's canal have been observed in clinical cases of siderosis and hemosiderosis of the anterior chamber. However, they were always found associated with obliterative changes in the trabecular meshwork. Hence, they may well be of secondary nature.

The different behavior of the blood vessels in the ciliary body and iris with regard to the permeability for SIO represents an unsolved problem. Electromicroscopic studies[17] reveal that the histological structure of the capillaries in the iris differs considerably from that of the capillaries in the ciliary body. However, these important observations do not offer a final answer to the problem. The differences in the staining behavior of iris and ciliary body cannot be explained by an absence of siderophilic substances in the iris. In fact, the iris takes up much iron when cross sections are stained with SIO.

The capacity for cytoplasmic iron uptake seems to be an inherent faculty of all endothelial and epithelial cells in the eye. By no means does this faculty appear to be restricted to endothelial cells of the blood vessels and phagocytes. The mechanism for the passage of iron through the cell membrane is not known. According to Richter,[3] electron microscopic evidence indicts pinocytosis as a principal mode of iron transport into the cell.

The clinical significance of the cytological changes induced by the iron in endothelial cells of the cornea and the trabecular meshwork, and the epithelial cells of the ciliary

body, the ora serrata, the pigment epithelium of the retina, and in the lens will be assessed elsewhere.[14, 16]

Summary

Experimental aspects of ocular siderosis and hemosiderosis following the intraocular or systemic administration of colloidal iron (SIO) or blood are presented. The major changes noted are either related to the uptake and binding of iron by the siderophilic ground substance of the vitreous body, of the perivascular tissue of the retina and optic nerve, and of the outflow channels of Schlemm's canal, or are based on the accumulation of siderin and hemosiderin in the cytoplasm of the endothelial and epithelial cells within the eye.

640 South Kingshighway (10).

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Discussion

Dr. Albert M. Potts (Cleveland): Dr. Cibis and Dr. Yamashita are to be commended for in this way amplifying our knowledge of the effects of iron compounds on the eye.

Because the human body contains grams of iron as hemoglobin and as liver deposits, it is too easily forgotten that iron is a heavy metal and, hence, toxic when the rather limited body mechanisms for disposing of ferric iron are exceeded. Iron combines with the carboxyls of protein and polysaccharide, as the authors mentioned, to alter tissue structure and interfere with the normal catalytic processes.

It is not much realized that there is a normally strong barrier in the intestinal tract against the entrance of dietary iron, and only enough iron is admited to fill current requirements; the rest is excreted directly.

When excessive amounts of iron are introduced into tissue by hemorrhage or a ferrous foreign body, there is a stimulus to the formation of apoferritin, an alpha-2 globulin. This protein can complex quantities of iron to make ferritin, which can hold as much as 23 percent of its dry weight of iron. When the capacity of this system is exceeded one finds clumps of pigment on microscopic sections

which look yellow-brown and which stain positively in the Prussian blue reaction, and which the histopathologists call hemosiderin. Examination with the electron microscope has shown in the past that these deposits are a mixture of ferritin (the specific chemical compound) and inorganic iron deposits which apparently are amorphous.

Here, as in many other similar cases, an entity which is perfectly clear to the histopathologist makes little chemical sense but makes a very great deal of medical sense. What the siderotic particles mean, practically, is that the tissue's capacity for handling iron is exceeded, and the tissue is in trouble.

How great the trouble is, varies with the tissue involved. If one is dealing with skeletal muscle or a piece of the liver, there may be no external sign of this difficulty at all. If one is dealing with the eye, even the tiniest lesion may mean industrial blindness. As the authors have shown, complete degeneration of neural elements of the retina; and, as they mentioned in their paper, loss of corneal boundary layers or proliferative changes in the retina may result from experimental siderosis.

One observation that seems particularly important to me is the observation on retinitis proliferans and the implication of this finding for the same condition in diabetes. These experiments indicate clearly that inorganic iron alone, without the added blood protein of diabetic hemorrhage, is an adequate cause for retinitis proliferans in the rabbit and possibly in the diabetic.

As you know, no one has consistently succeeded in producing experimental diabetic retinopathy. One wonders whether, with an inadequate stimulus, an insult of this iron injection type, which by itself would not be able to produce retinitis proliferans, if one might be able in a diabetic animal to induce such a change and consequently have another experimental tool for studying a difficult disease.

STUDIES ON CORNEAL TRANSPARENCY*

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Cleveland, Ohio

The problem of the interrelations between corneal hydration, corneal permeability to ions, and corneal transparency is one which has concerned visual physiologists for years and which in recent times has received an increased amount of attention.[1-3] The fact that corneal transparency decreases when corneal hydration becomes high has been considered axiomatic and is evident on simple inspection.[4,5] However, the exact quantitative relationship between hydration and transparency has not, to our knowledge, been measured, and it seemed of particular interest to us to consider transparency changes of the order of magnitude which is encountered in ordinary clinical experience. It is with the measurement of such changes that the present paper is concerned.

The measurement of corneal transparency presents numerous subtle problems, and the accomplishment of such a measurement in vivo would be most difficult indeed. For this reason, we chose to use excised corneas and performed in vitro measurements. Even here the definition of what should be measured is important. When an image-forming beam of light traverses the cornea, the image may be degraded by a number of independent processes: a portion of the image-forming light may be back-scattered from the cornea or it may be specularly reflected from the cornea. In neither case does the light traverse the cornea. An additional portion of light may be lost by absorption in the cornea itself. Still another portion may be lost by forward scatter and either escape the image area entirely or contaminate the image with forward-scattered light. As pointed out by Maurice, a large proportion of the light which escapes is scattered forward within a relatively small angle, so that without proper precautions gross experimental errors may be made.[3]

Fortunately for our purposes this same type of problem has concerned researchers

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in photography for years, since the image formed by light passing through a photographic negative is subject to the same degradation by forward scattering. Excellent treatments of the subject are found in chapter 20 of Mees' book on the theory of the photographic process,[6] and in a recent article by Weaver.[7] These writers review the classical concept of transparency as the ratio of transmitted to incident light, and the definition of density as the logarithm of the reciprocal of transmission. They also mention how early workers were forced to differentiate two types of density measurement: when all transmitted light, including scattered light, was collected—"diffuse density," and when only non scattered light was collected—"specular density." It is the measurement of specular density which gives the most accurate criterion of interference with image formation, and it is this quantity which concerns us in the case of the cornea. Our results are reported as D_s which is equivalent to $\log_{10} \frac{I_o}{I_s}$, where I_o equals initial intensity and I_s equals the intensity of transmitted light with the exclusion of scattered light.

Weaver published his measurements on a wide range of photographic densities and showed that, as density increases, the proportion of scattered light increases until, at high densities, virtually all of the light passing through the film is scattered. At low densities, those of chief concern to us, it is practical to measure specular density by restricting oneself to a very small angle of collection. One must compromise in such a situation between the angle ideally small for measurement and the erratic results that would be introduced by random irregularities in the small selected area of tissue.

We reached this compromise by collecting within a half angle of five degrees. To further reduce systematic error, we made the rough assumption that the type of scatter caused by cornea and by photographic image was of the same order of magnitude and utilized Weaver's data to derive correction factors for each density used. Only in a few instances did this correction factor reach 25 percent and in no case did it affect the qualitative picture.

Methods

The corneas used were obtained from beef eyes received one to two hours after the death of the animal. The corneas were dissected with a ring of sclera attached and held in the lucite holder previously described by us.[8] For density measurements, the lucite holder was placed in the photometer against an airtight gasket and the cornea inflated to a pressure of 25 mm. Hg. Measurements could then be made with only air in contact with the cornea; or, by placing a lucite window on either side of the holder, either endothelial or epithelial side could be covered with solution during the measurement. A collimated beam of light produced by an incandescent projection bulb and optical system was passed through the cornea and allowed to fall on a barrier layer photocell. This cell was masked except for a central area corresponding to the size of the original beam and subtending a half angle of five degrees.[8] After the initial density measurement, the cornea and holder were placed between two lucite half cells previously described[8] and allowed to remain in the surrounding medium of known composition for periods of time up to four hours. During this time the medium was stirred by an airlift stirrer with gas of controlled composition. In all experiments reported here, the gas mixture used was 95-percent: O_2 to 5.0-percent CO_2. Our experiments in the present series were done at room temperature.

At the end of the experimental period, cornea plus holder were removed from between the two half cells and placed once more in the photometer where density was determined a second time. The epithelium was then scraped and the transparency measurement repeated once more. In some experiments an additional attempt to evaluate

the type of contribution of the epithelium was made by placing fluid on the anterior and posterior surfaces and comparing measurements made in this manner with measurements made in air.

One series of experiments was done with equal osmotic concentrations on either side of the cornea. A second series was done where an osmotic difference was maintained between the two corneal surfaces. The absolute salt concentration on either side was varied as well as the concentration difference between the two sides. In other experiments, a hydrostatic pressure corresponding to 25 mm. Hg. was maintained on the endothelial side for the duration of the experimental period.

In a number of experiments, the wet weight of a central plug of cornea was determined and comparison made with the weight of similar plugs cut from control eyes before experimentation. In some instances, corneal thicknesses were measured by the careful use of micrometer calipers.

Because of the striking effect of potassium ion in reducing the transcorneal potential,[9] a series of experiments was done in which the bathing medium contained only potassium ion instead of sodium ion.

Results

The results of the experiments are presented in the following figures. Each experimental result represents a minimum of two corneas and in most instances more than two. In Figure 1 is shown the results of change of corneal transparency when corneas are maintained in a solution of sodium bicbarbonate at a pH of approximately 7.8 for 30, 60, and 90 minutes. The molarities of the bicarbonate solutions are shown beneath each bar. The density before and after scraping the epithelium is indicated for each time period. In each case there is an increase in density with time, but only in the case of the hypotonic 0.01 molar solution is there any sizable final density even after 90 minutes. Most striking of all, after denuding the epithelium virtually all of the obstruction to passage of light disappears; and in a few instances, the transparency of the stroma alone is greater than that of the initial intact cornea. This is shown by negative density figures on the graph; but negative or positive, these values are minute.

The effects of maintenance with asymmetrical concentrations are shown in Figure 2. Here the densities after short time periods were so low that the experiment was run for

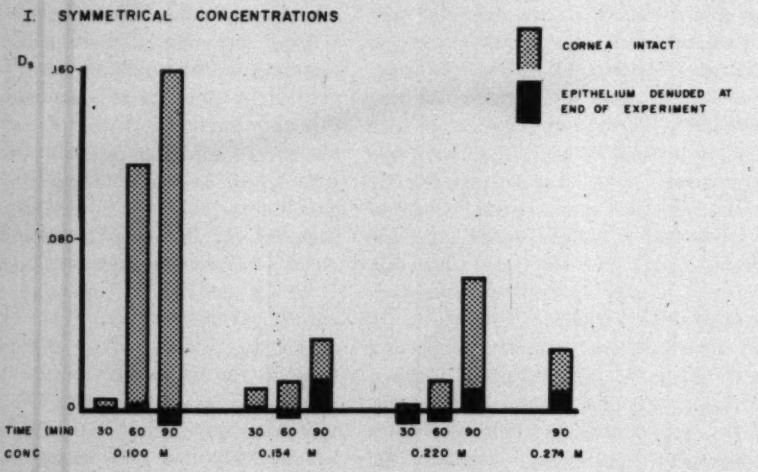

Fig. 1 (Potts and Friedman). Effect of osmotic strength on specular density of cornea.

four hours in each case. The higher concentration was always placed on the epithelial side; and it can be seen that once more, hypotonic solutions on the endothelial side make for increased density and that a minimum increase is reached in the third and fourth sets of results. The least increase in density seems to arise where a relatively large differential is maintained between anterior and posterior surfaces, such as in the third set of results and the last. In each case the epithelium again plays a large role, and transparency increases markedly after its removal. After four hours the contribution of the stroma begins to become significant.

Figure 3 shows the effect on transparency when the solution bathing the cornea is potassium bicarbonate instead of sodium bicarbonate. As can be seen from the results, less density develops in the cornea in the potassium solution in each case than in the corresponding sodium solution. The explanation for this particular finding is not a simple one, nor is the result the anticipated one. Here, again, for the 90-minute period or less, the chief contribution to density resides in the epithelium.

In Figure 4 is presented the effect of pressure on the endothelial side for periods of one-and-a-half and four hours. As can be seen, the density is very much increased by pressure for the four hour period; and, although only a third of this is attributable to the stroma, it still represents one of the high-

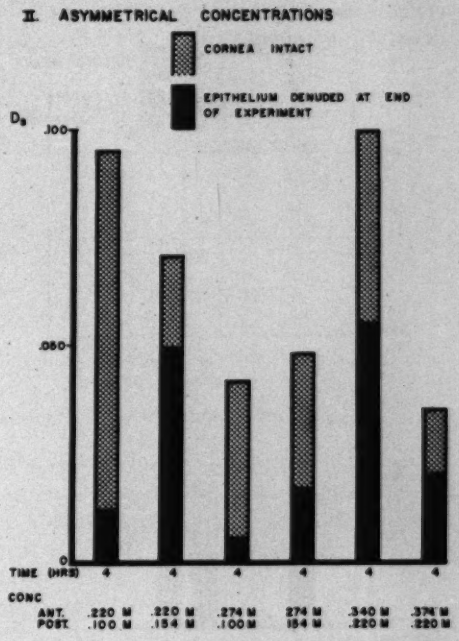

Fig. 2 (Potts and Friedman). Effect of osmotic strength on specular density of cornea.

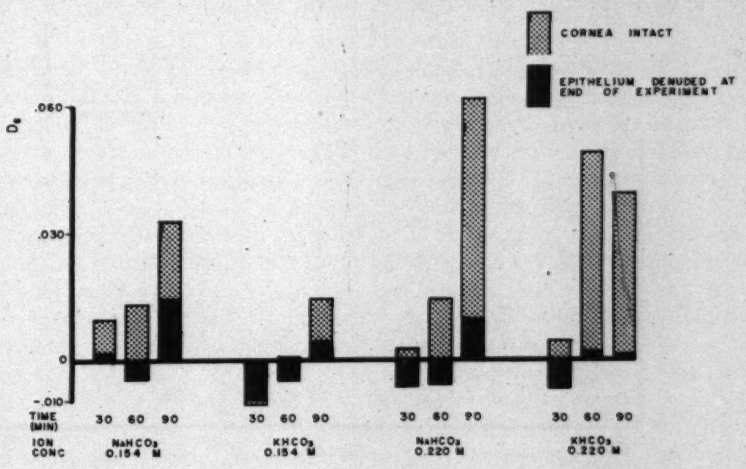

Fig. 3 (Potts and Friedman). Effect of K+ on specular density of cornea.

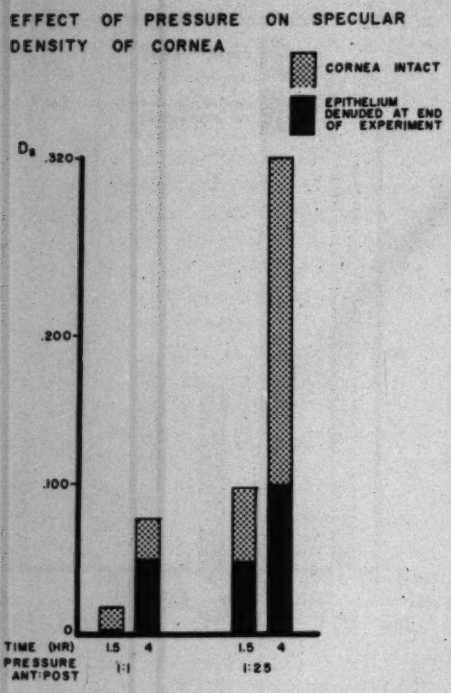

Fig. 4 (Potts and Friedman). Effect of pressure on specular density of cornea.

est stromal densities which we have measured in a four hour period.

In order to see whether there was a measurable water increase that paralleled the increase in density, the experiments where a central corneal plug was weighed and dried are reported in Figure 5. If the percent of the wet weight which is water is plotted against the specular density for each experiment, it can be seen that there is no trend and that increase in density does not bring a parallel increase in water content.

In Figure 6 where the results for density versus thickness are shown, once more there is no correlation between increase in thickness of the stroma and increase in density, although it is evident that there is an increase in thickness with time.

In order to get some notion of how densities of this order of magnitude affected human vision a simple test object was constructed consisting of sets of three black bars of equal width with spacing equal to bar width, placed on a transilluminated X-ray viewing box. The sets of three bars varied in size from 20 minutes to one minute of arc, corresponding to 10/200 to 20/20 vision. This test object was viewed through the cornea inflated in its holder and held before the emmetropic or corrected eye of the observer. It was found that the amount of turbidity required to reduce vision to resolving the 10/200 test bar was so slight that it did not register at all in our

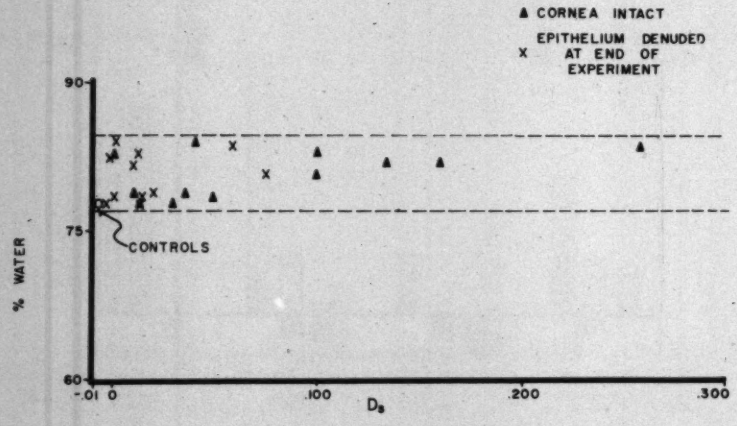

Fig. 5 (Potts and Friedman). Corneal water vs. specular density.

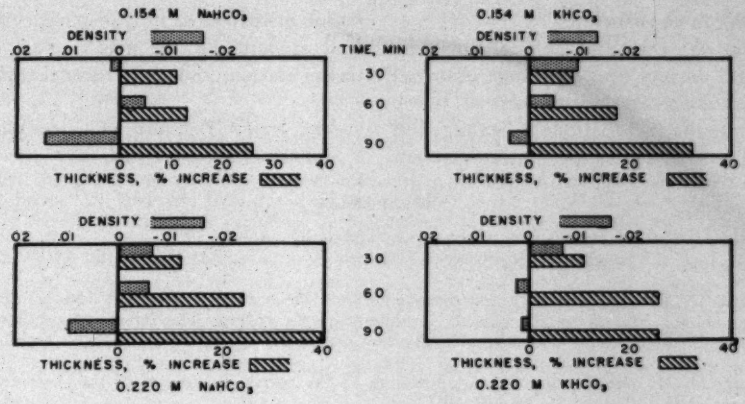

Fig. 6 (Potts and Friedman). Thickness of stroma vs. specular density.

densitometer. Hence, even with these modest densities of 0.3 at most, one is dealing with an obstruction many times worse than that which reduces vision to 10/200.

Discussion

The assumption is implicit in this work, as in all experiments on surviving tissues, that the observed phenomena have some relation to the processes taking place in the living animal. The very fact that density increases with time under our conditions is evidence of departure from normal in vivo behavior. We find it most remarkable that when this increase in density does occur, such a large portion is attributable to the epithelium. Although corneal irregularities can be seen by specular reflection, one does not see frank bullous keratitis, and one has the impression that diffuse cloudiness rather than surface irregularity is responsible for most of the effect. This is borne out by the fact that, although when the test chart is viewed through the cornea, acuity increases, when one puts an aqueous layer on either side of the cornea, there is little or no decrease in density measurable in the photometer.

The chief purpose of the experiments reported above is to establish a norm for the method on which the effect of experimental manipulations may be superimposed. Preliminary results suggest that many procedures known in the past to cause corneal hydration have as their first effect increase of density in the epithelial region. Further experiments in this direction are now in progress.

Summary

1. A method has been devised for measuring the specular density of the isolated beef cornea which is practical at least at moderate corneal densities.

2. The corneal density increases when the cornea is maintained in an environment of sodium bicarbonate, and most of this density is attributable to epithelial clouding. When asymmetrical concentrations of bicarbonate are used, the clouding occurs, but at a markedly slower rate; and optimal concentrations and concentration differences can be demonstrated. These approach those encountered in the actual eye.

3. Substitution of potassium ion for sodium ion causes less clouding in the same amount of time.

4. A pressure of 25 mm. Hg applied to the posterior corneal surface for the duration of the experiment causes marked increase in clouding compared to no pressure differential.

5. When one compares water content with density over the range observed by us no

correlation can be observed.

6. When one compares corneal thickness change with density over the range observed by us, there is no correlation observed.

7. Despite the fact that one is dealing with small amounts of hydration, these represent major disturbances in transparency and may well account for the greater part of ordinarily encountered early corneal turbidity.

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Discussion

Dr. John E. Harris (Minneapolis): This paper is another in a fairly extensive series by Dr. Potts on the cornea. He tackles it with his usual ingenuity. I must say that I am in essential agreement with his observations and his conclusions. The only thing I would like to do is perhaps restate them in a slightly different way.

Dr. Potts is concerned for the most part with the question of whether hydration is a big factor in corneal transparency. We all recognize, of course, that a cornea does not have to hydrate to become opaque. Loss of the regular lamellar structure and alteration of the critical distance between the fibrils, according to the theory of Maurice, will certainly cause a lack of transparency in an otherwise normally hydrated cornea. Irregular corneal surface and other factors may produce a similarly decreased light transmission.

At the same time and as Dr. Potts has stated, we must agree that when a piece of cornea hydrates to three to four times its normal weight it will become opaque. A relationship between hydration and opacity therefore does exist. Dr. Potts, however, is concerned with the twilight zone of a more minimal hydration and its relationship to corneal transparency. His preoccupation with this problem is sound, because this is the degree of corneal hydration that we see clinically.

Concerning the methodology which he has used, I can add nothing. He is measuring the light which is not forward-scattered. This is reputed to be a good measure of the image-forming ability of the optical structure under consideration and consequently is soundly used here.

As to his conclusions, I am not fully convinced that his data shows no relationship between transparency and hydration in the range he has studied. I wonder if I may have the next to the last slide of Dr. Potts', please.

(Slide) First one notices that the techniques employed which are known to most likely cause corneal hydration have the greatest influence on transparency.

Secondly, I wish Doctor Potts would tell us whether he has plotted this data in a slightly different manner. Here he plots transparency against percent water. This latter figure does not give a true picture of the hydration. Actually, the hydration could double, and the corresponding increase in percent water would be only 10 percent. For example, if we accept 77 percent water as the normal value and double the hydration the measured value as he charts it would be 87 percent.

I wonder therefore if Dr. Potts has plotted his data using the total amount of water per unit of dry weight as the ordinate. If he does that, I think that particularly with the data obtained with the intact cornea, he might find a more approximate relationship between degree of hydration and the lack of transparency.

Even so, this could not be explained on the basis of a stromal opacity. This is not surprising. Corneas do not swell regularly. Thus, slight swelling in a critical area may profoundly alter transparency. This would not permit the conclusion that even slight hydration has no influence on transparency

although it would modify any implication that the effect of hydration on corneal opacification results from stromal change. It is the irregular swelling of the cornea which is altering the transparency, and, as Dr. Potts has suggested, it might reasonably be placed at the epithelial layer.

DR. ALBERT M. POTTS (Closing): I would like to thank Dr. Harris for his cogent remarks. I don't think we are in any essential disagreement on the subject. We have not plotted the results in the way he stated, but simple inspection of the slide shows that the densities that are found in the region of no change in transparency have as much or more hydration than the densities that are toward maximum change.

This does not mean that there is no hydration of the cornea. All we claim is that there is no measurable hydration of the cornea, because, as we think, this hydration almost certainly occurs in the epithelium; and if the epithelium were hydrated to its total capacity we could hardly measure this in relation to the amount of water held by the stroma.

I am quite sure, too, that the epithelial irregularities are indeed the basis for much of the change in corneal transparency, because if we do the experiment described in the paper, of measuring the ability to see a test object across the room with the cornea suspended in air, as compared to the cornea where both layers are immersed in fluid (one can do this simply by taking the cell which held the cornea and putting windows on either side of it and filling the cell with fluid) then the ability to see such a test object goes up from the equivalent of less than 20/200 to something close to 20/25. However, when one measures transmission on the machine, this amount of change is still not measurable on our photometer.

So, in addition to the irregularity factor, there is also probably an epithelial clouding factor which has to be taken into account, and this again is different from the major stromal clouding factor which occurs in high degrees of hydration and which could be easily measured by determining water content.

THE EFFECT OF ARTIFICIAL OSMOTIC LOADS ON THE INTRAOCULAR PRESSURE*

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AND
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The most commonly accepted hypothesis for the mechanism of aqueous humor production postulates an active secretion of certain ions into the posterior chamber and the passive transport of water under osmotic forces. A certain amount of manipulation has been required in recent years to accommodate this theory to a growing body of knowledge. It is our purpose to report some new data applicable to the problem and to suggest that the importance of osmotic forces in the production of aqueous humor and in the regulation of intraocular pressure is considerably less than has been supposed.

* From the Department of Ophthalmology, University of Oregon Medical School. Supported by Grant #1979, National Institutes of Neurological Diseases and Blindness, Bethesda 14, Maryland. Presented in part before the Western Section meeting, Association for Research in Ophthalmology, November, 1957.

While we cannot replace the osmotic transport thesis with another well documented theory, the hypothesis that the aqueous is secreted as a unit, has a great deal of merit and is quite consistent with our data, and, we believe, with that of others. This is tantamount to saying that the movement of water across the blood aqueous barriers is under active control.

In a paper to be published elsewhere, we have been able to show that in a normal population of rabbits and cats, no discernible relationship could be found between the intraocular pressure and the aqueous-plasma osmotic difference. From this data it was evident that physiological variations existed in both functions. Furthermore, the physiologic variation in the intraocular pressure was considerably less than that of the osmotic difference.

In the present study, the aqueous-plasma osmotic difference has been subjected to more or less severe osmotic stress by artificial alteration of the plasma osmotic concentration. The effects on the intraocular pressure and on the aqueous osmotic concentration have been observed. Measurements were made under two basic conditions;

1. Using unanesthetized animals, a "spot check" of the intraocular pressure and the two osmotic samples, aqueous and plasma, were obtained within a very short time of each other at a particular time after imposing the plasma osmotic change.

2. Continuous recording of the intraocular pressure was done in anesthetized animals, running before, during and after the imposition of the osmotic stress. Plasma samples were obtained at intervals, and the experiment usually terminated by sampling the plasma and the aqueous from the unmolested eye. The terminal intraocular pressure and the osmotic pressure measurements then correspond to condition one except for the anesthesia.

We have interpreted this data in terms of the aqueous-plasma osmotic difference and the effect on this difference of the artificial stress. We have also interpreted it in terms of the relationship of the intraocular pressure to the aqueous-plasma osmotic difference.

Methods

Intraocular pressures were measured with a Hathaway Mod. P58A strain gauge communicating with the anterior chamber through a No. 50 polyethylene catheter and a No. 30 needle at the limbus. The electrical signal from the strain gauge was amplified and recorded with a Sanborn Strain Gauge Amplifier and Recorder. Throughout the calibration and measurement procedures, the strain gauge itself was not moved or handled. All manipulations were done with the needle at the end of the polyethylene catheter. The calibration was established in terms of the "head" in cms. of saline filling the catheter and needle. Calibration steps 10 cm. apart were recorded before and after each pressure measurement by moving the upper level of the saline column along a vertical meter stick. The effect of capillarity in the needle or catheter was eliminated by keeping the needle completely filled and its tip under the surface of sterile saline in a vial about 1 cm. in diameter. The precise zero of pressure was established by recording a short segment with the needle tip held in a pool of saline in the inner canthus of the eye immediately before inserting the needle into the anterior chamber. All measurements have been converted to mm. Hg STP, by means of a direct determination of the specific gravity of the saline. On numerous occasions erratic results were observed, presumably due to capillarity, when a bubble of air was allowed to remain in the needle or catheter. Therefore, means were provided with a syringe and a three-way valve to flush the catheter and needle with fresh saline before each measurement.

In "spot" measurements on unanesthetized animals, the head of the animal was stabilized if necessary by an assistant and the No. 30 needle inserted carefully under Pontocaine topical anesthesia. Then all restraining forces on the lids were released and the hub of the needle was carefully supported manually in such a manner that a minimum of distorting force was exerted on the cornea. Care was also taken to avoid any pressure on the orbits or the skin in the vicinity of the orbit. The initially high pressure recorded after the first penetration of the cornea and the advancement of the needle rapidly dropped as the animal became calm and any blepharospasm relaxed, usually within a half minute or so.

Figure 1 illustrates a typical record. In reading the records, the mean value of the lower excursions of the pen was approximated by eye. The assumption was made that any disturbance of the eye, needle pressure, blinks, etc. would cause the pressure to increase rather than decrease. When long con-

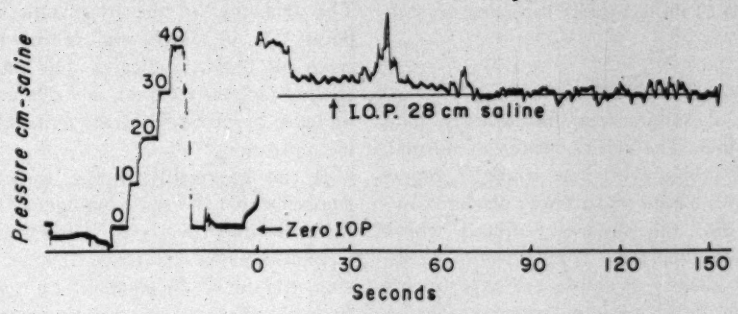

Fig. 1 (Tanner and Harris). Tracing of typical "spot record" of intraocular pressure. Calibration steps and zero base line determined with needle in saline pool in inner canthus are shown at left.

tinuous measurements of the intraocular pressure were made, the needle was balanced and suspended by an elastic band in such a manner as to avoid distortion of the cornea, and then left undisturbed throughout the experiment.

All experiments on rabbits were done with conscious animals under local Pontocaine instillation. The animals could be easily restrained, even for long periods, with little or no movement of the head by gentle pressure against the sides of the head. Short interval pressure measurements in cats were done under local anesthesia by having an assistant hold the head gently but securely. Long pressure recordings in cats were done under general anesthesia. Intraperitoneal Nembutal was most commonly used, but Pentothal and Chloralose were also used by the intraperitoneal route except for occasional small IV "booster" doses of Pentothal via the infusion needle.

Samples for osmotic measurements were always drawn from the eye not previously needled and the concentrations were measured with a Fiske Associates osmometer adapted for 0.2 ml. samples. 0.2 ml. samples of aqueous were drawn in oiled syringes and kept under oil during the measurements. Blood was obtained by left ventricular heart punctures in oiled syringes and centrifuged in dry heparinized centrifuge tubes under oil. 0.2 ml. aliquots were measured under oil with the osmometer in a like manner as was the aqueous. The osmometer was calibrated with NaCl solutions. Data from the International Critical Tables were used to apply activity coefficient corrections. That is, the data are reported in terms of an ideal solution, in water, 1.0 mosmol corresponding to a freezing point depression of 0.00187°C. Every effort was made to use a standardized technique for measuring both the samples and the calibration solutions. Multiple determinations were made on each sample and averaged. Some samples were measured more easily and with greater precision than others, but analysis of a typical example of a less favorable case gave a "confidence level" (by the t test) of about 85 percent that the reported value was within 3 mosmols and over 90 percent that it was within 4.0 mosmols of the "true" value. In cost instances the "confidence levels" are higher. Stated in another way, the average deviation from the mean of a small set of determinations on a single sample is usually about 2.0 mosmols and the maximum deviation of an accepted value in such a set is rarely over 4 mosmols.

Osmotic stress was applied to the blood-aqueous barrier by rendering the plasma hypotonic or hypertonic. In the latter instance, concentrated saline or glucose solutions were infused or injected into the vascular system. In the former case, hypotonic glucose was injected intraperitoneally. The slowly diffusing glucose served to keep a large fluid reservoir in the peritoneal cavity which depleted

the plasma of more rapidly diffusing crystalloids.

Results

Figure 2 summarizes the osmotic data from rabbits. The circles represent normal, untreated rabbit eyes. The triangles represent rabbits whose plasma was rendered hypotonic, and the squares, animals whose plasma was hypertonic from injection or infusion of glucose or saline. All experiments were done with conscious animals under local anesthetic. The solid line is drawn to indicate points of constant aqueous osmotic concentration, that is, it is the aqueous-plasma osmotic difference that would be obtained if there were no change in osmotic activity of the aqueous. It is drawn to pass through the mean value of the plasma and aqueous-plasma osmotic difference for the normal eyes. The scatter in these "normal" values is discussed in another paper. The points representing eyes subjected to osmotic stress across the blood-aqueous barrier are seen to lie relatively close to another straight line also drawn to pass through the mean point for the normal unstressed eyes. The fact that in the hypertonic cases the points all lie above, and in the hypotonic cases all below, indicates that the osmotic stress has been relieved, or "strained" by an increase or decrease in the aqueous osmotic concentration.

It can be readily shown, under the assumption that the stressing agent passes into the aqueous with a rate proportional to the aqueous-plasma difference, that is, by diffusion, and out of the aqueous at a rate proportional to the aqueous concentration, that is, by bulk flow, that such a straight line would be obtained when the stress is applied as a step function and the samples drawn at identical times. Spread of the points would certainly be anticipated since the alteration in plasma concentration undoubtedly was not a step function, and since the aqueous and plasma samples were drawn at times ranging from 16 to 30 minutes after beginning the plasma loading procedure. However, the data do appear to support in general contention that the imposed osmotic stress is relieved by a diffusion process, and that in the rabbit, the stress will be about 70 percent relieved in 20 minutes.

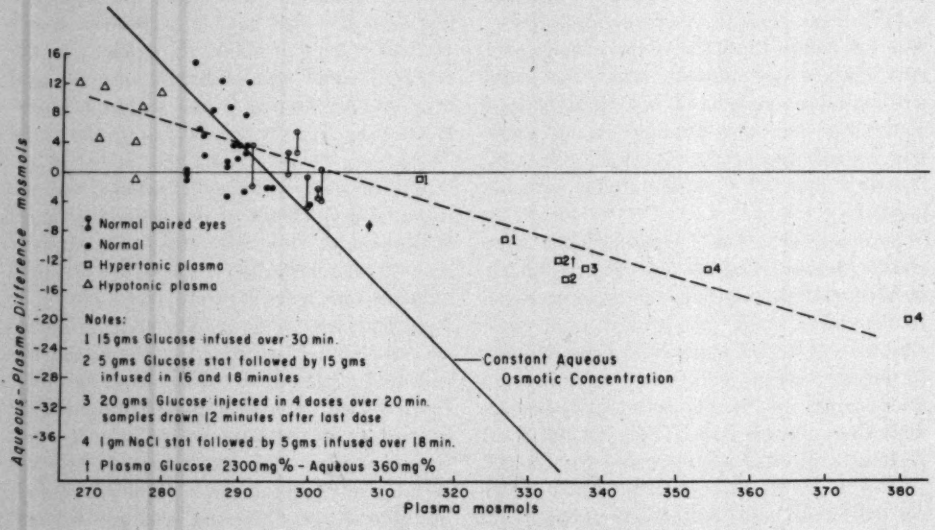

Fig. 2 (Tanner and Harris). Aqueous-plasma osmotic difference vs. plasma osmotic concentration for rabbits.

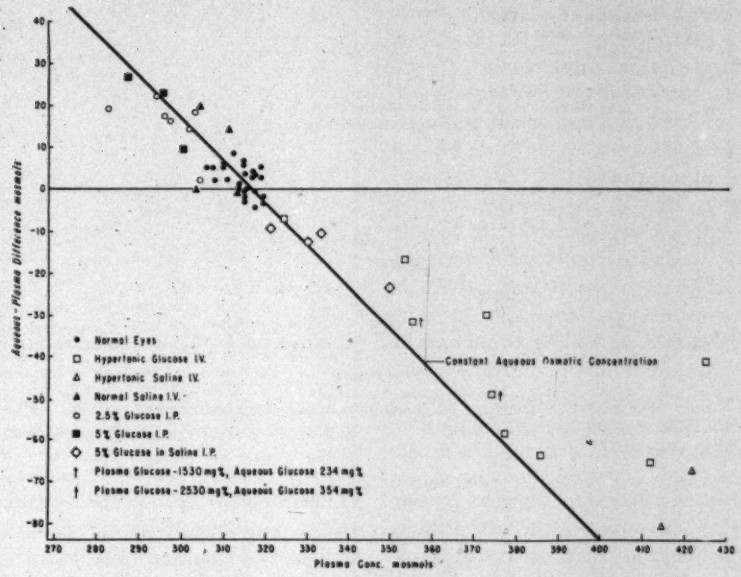

Fig. 3 (Tanner and Harris). Aqueous-plasma osmotic difference vs. plasma osmotic concentration for cats.

It is also interesting that there is very little difference between glucose and sodium chloride in this respect. In one experiment, using hypertonic glucose as a stressing agent, the aqueous and plasma were analyzed for glucose. While the plasma measured 2300 mg.%, the aqueous measured only 360 mg.%. In this case, although 65 percent of the stress was relieved, the passage of glucose could only account for about 35 percent. This is most easily explained as a reduction in the rate of secretion of water or as the resorption of water by the severe osmotic gradient resulting in the concentration of the other aqueous components.

The corresponding osmotic data from cats is summarized in Figure 3. Again the line of constant aqueous composition is included. It it evident that, in contrast to that of the rabbit, the osmotic pressure of the cat aqueous is little altered by the experimental manipulations. The fact that the cat has a large aqueous volume may be a contributing factor to this difference. However, it will be remembered that the normal cat produces aqueous at a considerably greater rate than the rabbit.[1] This and other evidence permits the conclusion that the difference in aqueous volumes does not account for the recorded species variation. The ability of the cat to maintain a relatively constant aqueous composition in the face of drastic changes in plasma composition has been noted before,[2] but it would seem worthwhile emphasizing the difference between animals in this respect.

Since most of the stressed points on Figure 3 represent experiments in which a continuous record was made of the intraocular pressure and the samples for osmotic analysis were drawn at widely varying times up to about 80 minutes after beginning the infusion or injection, no line has been drawn corresponding to the dashed line in Figure 2. However, the same general situation as regards relief, or strain, of the aqueous-plasma osmotic difference is evident from Figure 4. The percent of "strain" in this graph is a ratio the numerator of which is the vertical distance from the point in question on Fig-

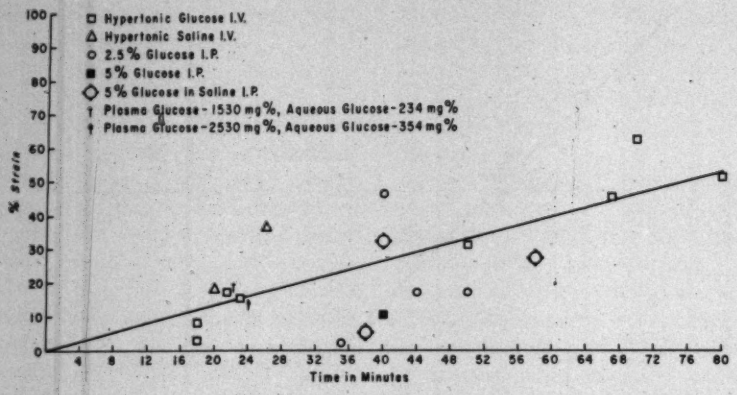

Fig. 4 (Tanner and Harris). Increase of aqueous-osmotic concentration in cat eyes under sustained osmotic stress. The straight line is intended to indicate a trend only and does not imply that the strain is actually linear with time.

ure 3 to the line of constant aqueous concentration and the denominator the total distance between the line of constant aqueous concentration and the horizontal line passing through the mean value of the aqueous-plasma difference in normal, unstressed eyes, in this instance, 2.1 mosmols. In other words, it is the fraction of the distance the point has "moved" from the 100 percent stress at the line of constant aqueous osmotic concentration to zero stress, which is taken to be the "normal" aqueous-plasma osmotic difference of 2.0 mosmols in favor of the aqueous. The time is measured from the beginning of the injection or infusion that is to establish the osmotic stress. Only points with plasma concentration below 300 mosmols or above 330 mosmols are plotted. Figure 4 with the straight line passing through zero is intended to indicate nothing more than the general trend of the stress relief with time and for comparison with the situation in the rabbit where the strain became approximately 70 percent in about 20 minutes. The most simple kinetic equations applicable would actually predict a parabolic type of rise in the strain function.

In two of the hypertonic plasma experiments with cats, the aqueous and plasma were analyzed for glucose. Interestingly, and in contradistinction to the situation in the rabbit, the amount of glucose passing into the anterior chamber more than accounts for the rise in aqueous osmotic concentration.

When hypertonic saline in infused into rabbits or cats, the result is invariably a decrease in the intraoscular pressure.[2] When the plasma is rendered hypotonic with respect to the aqueous, the intraocular pressure is increased.[3] The present work with rabbits essentially confirms the previous observations as is seen in Figure 5. According to Van't Hoff's law, 1 mosmol osmotic concentration difference is equivalent to 18.7 mm. Hg pressure difference. It is obvious that if the aqueous-plasma osmotic difference and the intraocular pressure were plotted in consistent units, the intraocular pressure would appear essentially constant for wide ranges of osmotic differences. In order to show the trend of the intraocular pressure, it is necessary to expand the pressure scale about 20 times. When this is done, there is clearly no direct proportionality between the intraocular pressure and the osmotic difference. Therefore, it must be concluded as it has before[4] that a static form of Van't Hoff's law does not describe the relationship between the intraocular pressure and the blood-aqueous osmotic difference.

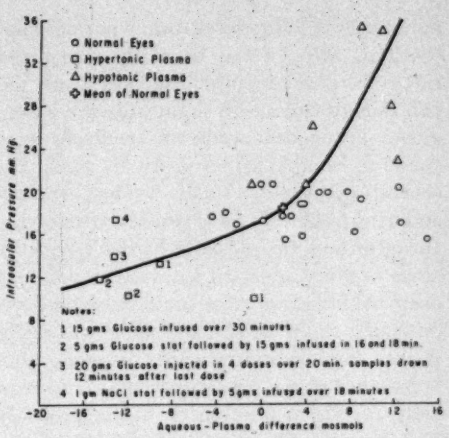

Fig. 5 (Tanner and Harris). Intraocular pressure vs. aqueous-plasma osmotic difference in rabbit eyes. The curve is a composite of two exponential curves joined at the mean value for normal eyes.

The scatter of the points on Figure 5 precludes any precise formulation of the actual relationship from the present data. However, the general trend seems to be similar to the pressure-volume curve of the globe[5] which is an exponential. To a first approximation, this is what might be expected if the effect of an osmotic difference in favor of the aqueous was to increase the volume of the globe. Actually, a simple exponential is a poor fit. The solid curve on Figure 5 is two exponential curves joined at the mean value point for normal eyes, obtained by fitting two straight lines to the points plotted on a semilong plot. The relationships between the rate of outflow and the intraocular pressure, and the scleral rigidity to the intraocular pressure, have not been exactly quantitated. Consequently, such a simple analysis may be misleading for anything but a general indication of the trend.

In similar experiments with cats, hypotonic plasma produced an increase in intraocular pressure and infusion of hypertonic saline produced a decrease, but infusion of hypertonic glucose appeared to produce an increase in the pressure. To observe this phenomenon in more detail, experiments were designed so that the intraocular pressure could be followed continuously, recording the normal intraocular pressure and all changes during and following the infusion. In a few cases, the femoral artery was cannulated and blood pressure simultaneously recorded. Of 15 experiments in which hypertonic glucose (25 percent) was infused, nine of the 12 studied by continuous recording showed elevations of 15 mm. Hg or more in the intraocular pressure, and two of the three in which only one measurement was made showed pressures substantially higher than normal. Of the four which did not show a significant rise, two were infused with 12.5 gm. of glucose or less (although the animal which achieved the highest intraocular pressure was also infused with only 12.5 gm.). These two and one other showed signs of poor anesthesia and a definite Nembutal effect was observed in both cases. The fourth animal was measured by a "spot check" with only local anesthesia and may well have experienced a higher intraocular pressure before the measurement was made.

The ability of barbiturates to lower the intraocular pressure has been observed for a number of years[6] and the present experience is in agreement. The injection of very small doses of additional Nembutal was several times observed to produce an immediate fall in the intraocular pressure which did not recover. This was observed both in the two cases mentioned where the pressure failed to rise with glucose and also as a later event in several instances where the pressure did rise. These Nembutal injections were almost all made to deepen the anesthesia when the animal appeared to be reacting to painful stimuli. Since under smooth conditions the Nembutal did not prevent the rise in intraocular pressure upon infusion with glucose, it may well be that other nervous system factors must combine with the barbiturate to block or reverse the rise in pressure. However, it is our feeling that deep barbiturate anesthesia by itself will prevent the hypertensive action of glucose. Chloralose, and to some

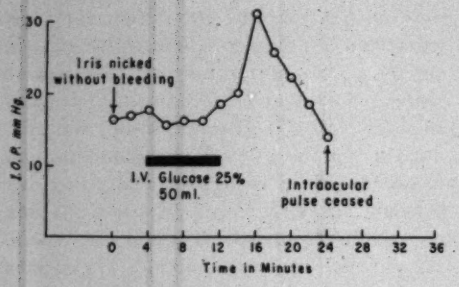

Fig. 6 (Tanner and Harris). Continuous record of intraocular pressure in a cat eye showing effect of glucose infusion and mild trauma to the iris. The needle had clotted at the point where the intraocular pulse was no longer recorded.

extent Pentothal anesthesia seemed to have less effect. Nevertheless, stormy induction or retching was rarely crowned by success in elevating the intraocular pressure regardless of the anesthesia.

Mechanical disturbance of the iris is a well recognized hazard when continuous recordings of the intraocular pressure are being made. Two observations in the present work are relevant to this. If the iris is perforated, nicked, or sometimes even only touched by the needle, the pressure rise is prevented or, if already elevated, a rapid fall is produced. On occasion, if the trauma is very light, some rise is allowed followed within several minutes by a fall to levels below normal (fig. 6). This artefact can be easily recognized since the normal pulse disappears and the pen fails to move with slight pressure on the globe. When the needle is removed, it is found to be occluded, evidently by a clot. On several occasions without evident trauma and after relatively long periods of uneventful recording, the pulsations would suddenly cease and the apparent intraocular pressure drop. Although no clot could later be demonstrated on the needle, and saline was easily flushed through it afterwards, it seems quite apparent that the needle served as a foreign body for the deposition of fibrin, probably in a thin film. This implies that slight trauma to the eye, iris, or cornea, or perhaps only from the elevated intraocular pressure, caused the slow perfusion of plasma proteins into the aqueous. Recent work on the reduction of the intraocular pressure by humoral factors liberated by elevated intraocular pressures[7] may be applicable here.

It was noted that often the intraocular pressure would slowly drop from its highest value, ultimately to levels below normal, and further infusion of glucose would not reverse the trend (fig. 7). Systemic trauma, as from the surgery of cut-down to artery or vein or even heart punctures also appeared

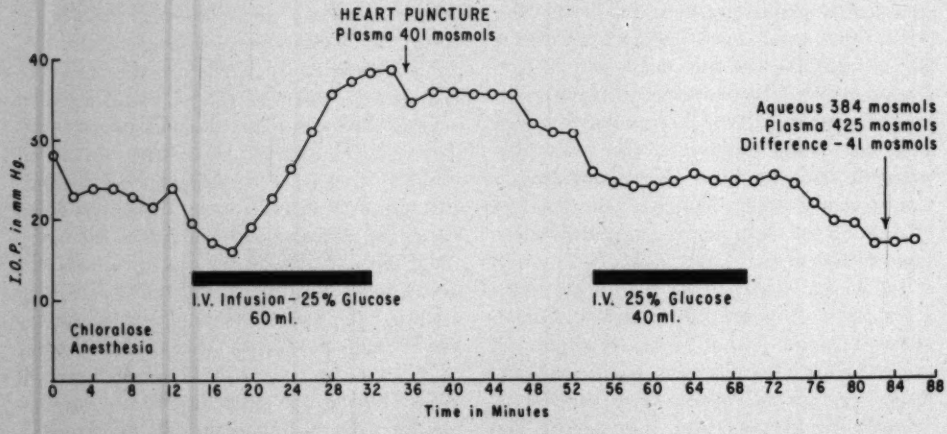

Fig. 7 (Tanner and Harris). Continuous record of intraocular pressure in a cat eye showing hypertensive effect of initial glucose infusion and gradual decline not reversed by additional glucose infusion.

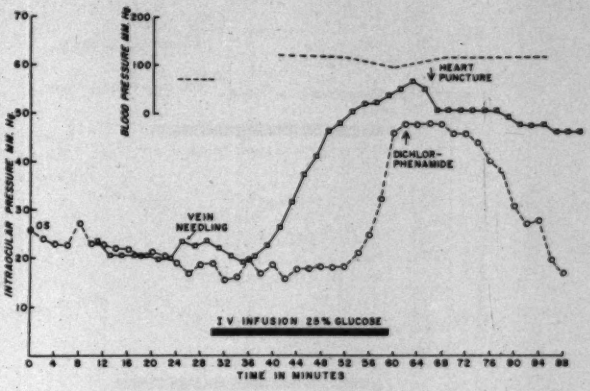

Fig. 8 (Tanner and Harris). Two superimposed continuous records of intraocular pressure in cat eyes showing hypertensive effects of hypertonic glucose infusion, effect of mild trauma, and effect of carbonic anhydrase inhibitor (dichlorphenamide). The blood pressure recording obtained simultaneously with the latter record indicated essentially constant blood pressure during elevation and subsequent drug induced decline in the intraocular pressure.

to predispose to or to precipitate early fall in the elevated intraocular pressure and to occlusion of the needle. It is possible that similar humoral factors are released by damage to tissues distant from the eye which tend to break down the blood-aqueous barrier.

The effect of ocular hypotensive drugs is shown in Figure 8. In this experiment, in which the blood pressure was also recorded, the carbonic anhydrase inhibitor dichlorphenamide was injected, 15 mg. per kg. at the time the glucose infusion was stopped. Within ten minutes, the intraocular pressure had started to fall and continued to do so. During the manipulations attendant on cannulating the artery and establishing the intravenous infusion, the average blood pressure tended to be low, about 70 mm. Hg. Shortly after beginning the infusion, after all manipulations had ceased, the blood pressure rose to about 120 mm. Hg. While the intraocular pressure was rising rapidly, the average blood pressure fell slightly to about 96 mm., then rose back to 116 to 118 mm. as the intraocular pressure stabilized and began to fall.

Superimposed on Figure 8 and drawn with a solid line, is shown the result of a smooth experiment with no drug or other treatment in which the intraocular pressure reached one of the highest levels observed in the present series. The slight perturbing effect of establishing the percutaneous intravenous infusion is shown, as well as that due to a heart puncture at the peak of the pressure rise. In general from our experience, it can be said that mildly perturbing trauma at pressure normal or below tended to raise as well as lower the immediate pressure, while trauma at elevated pressures always lowered the pressure. If the perturbation was small, the pressure might rise again, but usually it was permanently reduced in the stepwise fashion shown.

Figure 9 illustrates control experiments and shows the negligible effect of infusing isotonic saline and the essentially negligible effect of infusing isotonic glucose.

Since all our previous work on rabbits had demonstrated a reduction in intraocular pressure when infused with hypertonic solutions, glucose as well as saline, it was at first thought that rabbits would not show the anomalous glucose effect. However, when the experiment represented by Figure 10 was performed, it was evident that this animal too shows the effect although to a much less degree. Under local anesthesia, the intraocular pressure recording was started and allowed to run a short time. Then 10 ml. doses of 50 percent glucose were slowly injected into the left ventricle. The first two doses clearly resulted in a rise in the intraocular pressure, but the third and fourth doses resulted in sharp decreases which con-

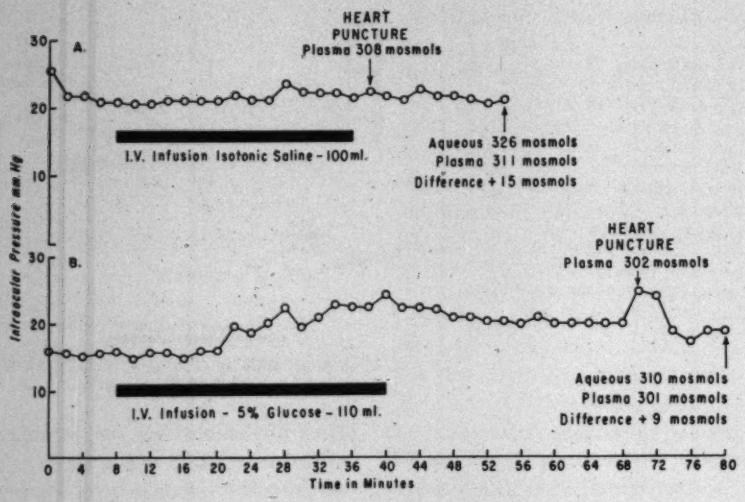

Fig. 9 (Tanner and Harris). Two control experiments showing the negligible effect on the intraocular pressure in cat eyes from infusion of isotonic saline and glucose.

tinued to lower than normal levels. This would appear to be another illustration of the lability of the blood-aqueous barrier in this animal and the seemingly greater osmotic transfer of water.

A number of continuous records were obtained of the effects of hypotonic plasma from 2.5 percent and 5.0 percent glucose injected intraperitoneally in cats. Two of these are shown in Figure 11. In general, the effect was to raise the intraocular pressure, subject to the perturbing effects of trauma as noted above (fig. 11-curve A). Dichlorphenamide always produced a sharp drop as in Figure 8, and if injected beforehand, prevented the subsequent rise (fig. 11-curve B). There is some question about the mechanism of the rise in these experiments. It may be that glucose diffusing out of the peritoneum is primarily responsible. This is born out by the general impression that 5.0-percent glucose is more effective than 2.5-percent solutions. One would expect that the isotonic solution would deprive the vascular system of water as well as salt and tend to hold the pressure down while the hypotonic solutions would tend to increase the vascular volume and help the pressure rise. This does not seem to be the case. In two of eight experiments in which 5.0-percent glucose in

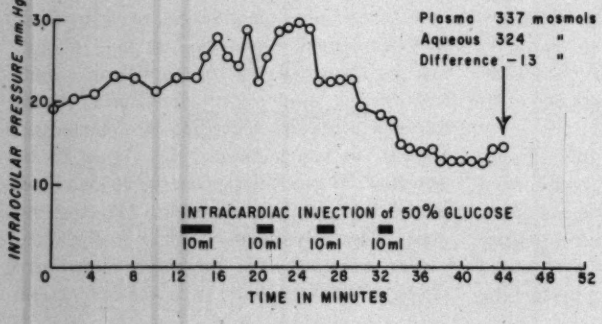

Fig. 10 (Tanner and Harris). Continuous record of intraocular pressure in a rabbit eye showing hypertensive effects of initial glucose injections with subsequent fall after additional injections, demonstrating the greater fragility of the blood-aqueous barrier in this animal as compared with the cat.

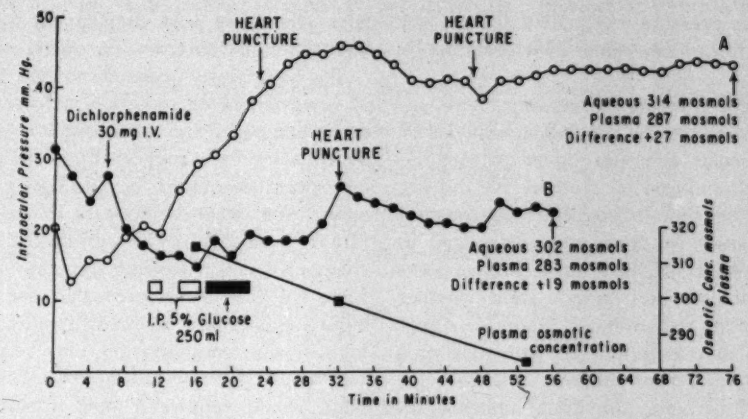

Fig. 11 (Tanner and Harris). Two continuous records of intraocular pressure in cat eyes showing hypertensive effect of hypotonic plasma induced with intraperitoneal injection of isotonic glucose and the blocking of this effect by previously injected carbonic anhydrase inhibitor (dichlorphenamide). The steady decline of the plasma osmotic concentration produced by IP isotonic glucose measured simultaneously with the IOP record of curve B is shown.

normal saline was instilled in the peritoneal cavity, the intraocular pressure rose markedly. In one of these cases the intraocular pressure rose to a maximum of 42 mm. Hg and was sustained over 32 mm. for over half an hour before the needle clotted. The plasma glucose measured 312 mg.% at this time. In the other six of these experiments with glucose in saline, the intraocular pressure showed little or no change as would be expected.

Comment

One of the inescapable conclusions from the data here described is that osmotic equilibration with respect to water is considerably slower than would be anticipated from measurements of the rate of exchange of deuterium or tritium labeled water between plasma and the anterior aqueous.[8, 9] This is true in the rabbit and is particularly striking on the cat. The recently published observations of Bárány[10] and Auricchio and Bárány[11] on the movement of water across the blood-aqueous barriers of the cat under an osmotic load are in essential agreement. We can disallow on a quantitative comparison the thought that this species difference is due to a difference in aqueous volumes. Moreover, it will be recalled that the turnover rate of various substances in the anterior aqueous is essentially the same in the two animals. (Comparative data can be found in the excellent book of Davson.)[12] The discrepancy is particularly intriguing when it is noted that in other systems the permeability of living membranes to water is greater when measured by osmotic flow than with diffusional exchange of deuterium oxide[13] although admittedly our data cannot be directly compared with these observations.

One explanation of the apparent discrepancy we have observed which must always be considered is the possible exchange of deuterium or tritium with hydrogen from water or other molecules. This is known to occur rapidly.[14] Thus the published values for turnover time of labeled water may in truth at least partially represent an exchange of hydrogen.

In the discussion which followed Bárány's paper[10] the suggestion was raised that under the imposed osmotic load the formation of aqueous might be reduced. This may be true but such a suggestion does not explain the observed failure particularly in the cat of

the osmotic pressure to equalize. Clearly, an active control of movement of water must be exerted.

The most striking observation here reported however, is the anomalous behavior of the intraocular pressure under plasma osmotic loads induced by glucose. We did not measure the flow of aqueous at constant pressure since we were more interested in the hydrostatic pressure under differing osmotic gradients and cannot state whether this pressure rise induced by glucose was due to an increased aqueous formation or a decreased facility of outflow. However, the fact that a carbonic anhydrase inhibitor, dichlorphenamide, prevented the rise would suggest that the bulk flow of aqueous was increased in spite of the fact that the plasma was hypertonic with respect to aqueous.

The ability to induce this anomalous pressure rise was restricted to glucose and was not observed when hypertonic saline was used. The reason for this effect of glucose is not understood. We are inclined to believe that it is associated in some manner with the glucose plethora presented to the ciliary epithelium. This on the surface seems at odds with the well known observation that in diabetic coma the ocular tension may be lowered. However, this is an abnormal situation and probably more related to the insulin deficiency. The influence of insulin on aqueous formation in general has not been adequately studied although Ross has demonstrated an insulin dependent glucose transfer across the blood aqueous barrier.[15]

The observation that a glucose plethora may alter transport is not new. We have observed an interference with cation transport across the lens surface exposed to a high external concentration of glucose.[16]

We have always considered that the rate of involvement of some key substance may determine the rate of formation of bulk aqueous and have suggested that the particular substance might be glucose.[17] Dibenamine, for example, appears to reduce the rate of transport of glucose into the anterior chamber.[18] As a working hypothesis, therefore, we have considered that the normal ciliary epithelium, when confronted with a high glucose concentration, may respond by increasing the rate of aqueous formation. This would require a bulk formation of aqueous possibly in the manner described by the term pinocytosis. Superimposed must be a control of water across barriers where one would normally expect a ready osmotic flow.

Summary

1. The influence of hypertonic and hypotonic plasma osmotic loads on the aqueous-plasma osmotic pressure difference and the intraocular pressure has been measured in the rabbit and cat.

2. The rate of osmotic equilibration in both animals but particularly in the cat was less than would be anticipated from the previously measured exchange of labeled water.

3. Hypertonic glucose solution produced an anomalous intraocular pressure rise in the cat.

4. The bearing of these findings on our present knowledge of the mechanisms of aqueous formation are discussed.

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Discussion

Dr. Elmer J. Ballintine (Cleveland): Several features of Dr. Tanner's work seem most significant. First, he has developed a technique for direct cannulation of the anterior chamber of cats and rabbits, anesthetized and unanesthetized, which produces minimal disturbance of intraocular pressure and results in the consistent maintenance of intraocular pressure for at least an hour and a half.

The experience in previous investigations with which I am familiar was that cannulation, in vivo, is most frequently followed by one or more nonphysiologic sequellæ such as falling intraocular pressure, production of plasmoid aqueous, and obstruction of aqueous drainage by fibrin clots. Herparinization and other complicating details have not notably improved consistency in the past.

I would like to know particularly if Dr. Tanner's technique of cannulation does result in aqueous humor that is similar to normal rather than plasmoid aqueous in most of the eyes. I would appreciate any data on ascorbic acid, and bicarbonate, which are the obvious substances that can be determined easily. The development of a technique which permits cannulation with minimum disturbance of aqueous humor dynamics and composition is an achievement that has been long awaited by everyone working in this field.

The second important development is the observation that in the cat the administration of glucose by several routes produces a sustained elevation of intraocular pressure. The authors favor the interpretation that the glucose produces an increase in the production of aqueous humor. Although the rate of production was not measured, it can be estimated approximately on the hypothesis that the significant effect of the glucose was to increase aqueous humor production without changing the coefficient of outflow.

To maintain the intraocular pressure at 50 mm. of mercury in the cat, as was shown in the dichlorphenamide experiment, would require that not less than 50 microliters of aqueous humor per minute be produced. This is at least a four-fold increase over even the highest rates that have been observed in the past.

Such an increase in flow was not found by Bárány in cats in experiments similar to these, using hypertonic fructose. I would like to see results of experiments similar to these in which the eye is maintained at constant pressure with provision for measuring the rate of efflux of aqueous after glucose administration. Such experiments would show whether most of the pressure rise was the effect of change in coefficient of outflow or whether it was the change in the rate of production.

If a facilitated transfer of glucose into the posterior chamber is the cause of the pressure elevation, an attempt to abolish the effect with phlorhizin might throw some light on the mechanism of the transfer.

Finally, I would like to introduce a slight degree of skepticism in the interpretation of small differences of osmotic pressure in plasma and aqueous humor. There are a number of objections

that could be raised, and these are two fairly obvious examples.

The pH of aqueous differs from that of plasma, yet the specimens are measured in an osmometer where they tend to equilibrate with an atmosphere of air. I feel reasonably sure, if the specimens were again measured in an atmosphere where they tended to equilibrate with alveolar air, the osmotic differences would be altered.

The activity of ions, especially in the plasma, will be altered by mild changes in the plasma protein, brought about by the loss of CO_2, by temperature changes, by heparinization and by centrifugation. These, are only a few of the things that could be imagined to affect the measurement of small osmotic pressure differences.

DR. K. NOLEN TANNER (closing): Thank you Dr. Ballintine for your remarks. Our success in making sustained records of the intraocular pressure is not unique, and I believe we use about the same technique as others. We use the smallest needles, No. 30, and make every effort to avoid touching the iris and to make the puncture as atraumatic as possible. Much of the work reported in the literature was done with rabbits and I have no doubt that these animals are more difficult to work with in this respect. Cats seem much better able to maintain normal intraocular pressure in the face of disturbance.

We have been aware of the possible criticisms of the accuracy of our reported osmotic measurements. We did draw both plasma and aqueous in oiled syringes and performed all necessary manipulations under oil. The 0.2 ml. sample of aqueous was transferred once from the syringe to the measuring tube under a standard layer of oil about 2 mm. thick. Since the plasma was centrifuged twice, several transfers were necessary, so to minimize carbon dioxide loss fairly substantial quantities, 5 to 10 ml., were drawn from the animal and handled under 5 to 10 mm. of oil. For the osmotic measurement, 0.2 ml. were drawn up into an oiled syringe and injected under oil exactly as was the aqueous. In a few tentative experiments in which the measurements were made without the oil, I received the impression that there was little or no change in osmolarity which might be ascribed to carbon dioxide loss. However, the behavior of the cooling curve of a two phase system such as ours is very sensitive to the heat capacity of the various components and to the rate of heat exchange when the sample size is very small. Consequently it is necessary to standardize carefully all parameters of the technique. The study of our raw data leads me to feel that our results are precise within 2 or 3 mosmols and I doubt that carbon dioxide exchange with the air has effected them more than that.

We are grateful to Dr. Ballintine for suggesting the interesting further experiments. We intend to try other stressing agents like fructose, and also the effect of insulin and antimetabolites. The rate of flow of aqueous is, of course, crucial in the interpretation of data such as ours. We have been studying a modification of conventional tonography which may enable us to determine this factor.

NUTRITIONAL AND ENDOCRINE INFLUENCES ON THE SYNTHESIS OF ALBUMINOID IN RAT LENSES*

I. THE EFFECT OF RESTRICTED DIET AND L-THYROXINE ON THE ALBUMINOID FORMATION

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Results of previous investigations on synthesis of proteins of rat lenses during the development of experimental cataracts showed that the ratio of the albuminoid to the soluble proteins increases at early stages of cataract formation.[1, 2] It has been known for a long time that during the development of senile cataracts in man the relative content of lenses in albuminoid increases with progressing cataract. More recently it has been shown that this is the case also in senile cataracts of beef, horse[3] and rats[4] as well as in partial human cataracts.[5]

These findings suggest that an increased albuminoid formation might be at least one of the pathogenic factors in the mechanism of cataract formation. It seems reasonable, therefore, from this point of view to assume that factors which are able to influence the rate of albuminoid formation might play a role in the etiology of cataract.

From the chemical point of view the formation of albuminoid was shown to be associated with the appearance of a large proportion of cystine in lens proteins, an amino acid which does not occur in significant

* From the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University. This work was supported by a U. S. Public Health Service Grant number B-1142.

amounts in soluble lens proteins. The albuminoid synthesis, therefore, can be assumed to be linked to a slow oxidative process in the lens, and an increase of the synthesis can take place whenever oxidation of lens proteins is accelerated either by an increase of the oxygen reduction potential inside the lens, or by an increase in the susceptibility of lens proteins to oxidative agents.[4] The role of the latter factor appears of particular interest as one part of cysteine in soluble proteins had been shown to be inaccessible even to strong oxidizing agents.[4] In addition to such local factors, however, other factors must be taken into consideration which are able to regulate oxidative processes and protein synthesis not only locally, but in the whole organism.

One of such systemic factors which is known to be associated with albuminoid formation is the aging of the organism and of lens fibers. It has been known since the classical investigations of Mörner that the albuminoid increases at the expense of soluble protein with increasing age of the whole organism and of the lens proteins. It seems possible, therefore, that certain systemic conditions which can be shown to be related to the aging of the whole organism could also influence the formation of albuminoid in the lens in the same sense as they are able to influence the aging process in the whole organism.

As the maximal life span of a species seems to be a hereditary trait the acceleration of retardation of the aging process will express itself in shortening or prolongation of the life span of an animal. There is some suggestive, though far from conclusive evidence, that the rate of the maintenance of metabolism of the whole animal may be related to the average life span of the animal. The fact that the life of cold-blooded animals is shortened by an increase in the temperature of the environment can be interpreted in this way.[5]

More than 50 years ago Max Rubner[6] suggested that certain classes of animals, in the total amount of energy spent per gm. body weight during the lifetime of the animal is nearly constant for various species of the same class of animals. He reported data according to which the total amount of energy spent per gm. by resting domesticated mammals during their maximal lifetime is almost equal for various animals and this appears to be true in such extreme cases as guinea pigs and cattle.

On the other hand, McCay[7] and his associates reported, in 1935, that when rats which are a few weeks old are kept on a diet which provides adequately for proteins and vitamins, but is calorically deficient, they can be kept for a prolonged period of time at a constant weight and can be prevented almost completely from growing. McCay showed that those animals which under these conditions survived an increased mortality due to higher susceptibility to infections, lived up to 50 percent longer on the average than control animals on calorically adequate diets. As the metabolic turnover of these animals on the calorically deficient diet undoubtedly can be assumed to be decreased as compared with that of controls, the prolongation of the natural life span in these animals could be at least in part related to the lowering of the metabolic rate.

It seemed possible, therefore, to expect that such systemic factors which increase the metabolic turnover of the organism, or restricted diets which prolonged the life span of the animal might influence the synthesis of albuminoid in the sense that it might be increased with the rising and decreased with the lowering of the metabolic rate. The present report deals with two series of experiments in which the influence of thyroxine and restricted diet on the albuminoid formation in lenses of four to five-week-old rats was investigated.

Experimental

I. General procedure

In general experiments were carried out in such a way that two groups of 10 animals each were put under experimental conditions,

whereas two groups of the same size were kept under standard conditions. A fifth group of ten animals was sacrificed at the beginning of the experiment to determine the initial value for the amount of soluble and insoluble proteins in their lenses. The age of the animals at the beginning of the experiment was either 28 or 35 days. At the end of the experimental period the animals were killed with ether, the lenses removed immediately, wiped off with filter paper, and weighed in a weighing bottle. Lenses from each of the two experimental and control groups were pooled together, and homogenized in an Elvehjem-Potter homogenizer in a 10-fold amount of their weight of distilled water. The homogenates then were centrifuged at 10,300 × g for one hour in a refrigerated centrifuge and the supernatant removed. When the centrifugation of the supernatant was continued for another hour, no insoluble residue could be observed. The insoluble residue remaining after centrifugation was twice washed with distilled water, once with saline, and then twice with 5% trichloroacetic acid (TCA). One cc. of the supernatant was diluted with four cc. of H_2O and precipitated by adding 0.1 cc. of 100 percent TCA. The precipitate was washed with five-percent TCA and dissolved by filling up to 10 cc. with 0.5 N NaOH. Nitrogen determinations on both these solutions were carried out by micro-Kjeldahl in duplicate. The maximum difference between the duplicates was less than two percent. The averages of the two determinations were used for the calculation. The amount of soluble proteins per lens was calculated by multiplying the amount of nitrogen found in one cc. of the solution by the volume of the homogenate, the volume of lenses being calculated from their weight and density of one, and dividing it by the number of lenses which were homogenized. The protein content was calculated by multiplying the nitrogen content by the factor 6.25. The same procedure was used for the calculation of the amount of albuminoid per lens.

Results

A. The influence of restricted diet on the formation of the albuminoid

Five experiments on the effect of food restriction on the synthesis of albuminoid and soluble proteins were carried out. In all experiments the two groups of control animals in each experiment were allowed to eat ad libitum, whereas the two groups of experimental animals were given only a significantly smaller amount of the same food. To prevent that some of the animals would eat much more of the restricted diet than the others, all the experimental animals were kept separated in individual cages and the restricted amount of food was offered separately to each of these animals. In four of the five experiments the diet consisted of the usual Rockland Farm pellets, of which the control animals ate on the average 12 gm./rat/diem, whereas the experimental animals got in the first three days 8.0, and in the following period only 7.0/gm./rat. Of four experiments of this type, in two the experimental period extended for 20 days and the age of the animals at the beginning was 35 days. In the two others the experimental period extended to 44 and 60 days respectively, and the age of the animals at time zero was 28 days.

In the fifth experiment the diet was prepared according to McCay and his associates,[7] as follows:

Starch 22 percent, cellulose two percent, lard 10 percent, sucrose 10 percent, salt mixture six percent, dried yeast five percent, cod liver oil five percent, and casein 40 percent. Of this diet the control animals consumed about 12 gm./rat/diem, whereas only 5.0 gm. were offered to the experimental animals. According to McCay and his associates, this diet even when consumed in quantities so small as to inhibit almost completely the growth of the rats, contains still sufficient amounts of proteins and vitamins and is only deficient as far as calories are concerned.

In this last experiment the experimental period lasted for 49 days. The results of all five experiments are presented in Table 1. As can be seen in experiments with the restricted pellet diet, the growth of the animals as far as body weight is concerned was almost completely suppressed during a period of up to 60 days. It was not possible, however, to achieve as high an inhibition of the increase of growth with McCay's diet, although the caloric restriction in this case was much higher than in the other case. It didn't seem reasonable to lower still more the food intake in this experiment as under this regime the animals seemed to be extremely restless and aggressive, which created some difficulty in feeding.

As can be seen from Table 1, the amount of albuminoid formed during the experimental period was lower in all 10 experimental groups in the five experiments, and whereas the total amount of albuminoid was 10 to 20 percent lower in the experimental animals than in the controls, the increase in the synthesis calculated by dividing the amount of albuminoid formed during the experimental period (by subtracting the amount at the end of the experimental period from the amount at time zero) was up to 27.7 percent lower than in the control animals. At the same time the food restriction inhibited the synthesis of soluble lens proteins in four out of five experiments. This inhibition is accomplished in most cases by a slight decrease in the wet weight of the lens, which however, in no case exceeded five percent of the lens weight of controls.

B. The influence of thyroxine injections on the synthesis of lens proteins

Six experiments of the same kind were carried out in which the effect of injection of 0.5 mg. of the Na salt of L-thyroxine per 100 gm. body weight on the synthesis of albuminoid and soluble lens proteins was studied. The thyroxine was injected once a day subcutaneously and the animals kept on the standard pellet diet. In three experiments the experimental period extended over 18 days, in three others, over 10 days.

The results are listed in Table 2. As can be seen, the animals which received thyroxine injections increased their weight to a much lesser degree than the controls. In all five groups of experimental animals there was a significant increase in the albuminoid content of lenses as compared with the 12 control groups. The soluble proteins, on the other hand, showed a significant marked decrease. This decrease was percentually stronger than the simultaneous decrease in the wet weight of the lens. The rate of synthesis of albuminoid was very markedly increased between 30 and 50 percent as compared with controls. The statistical evaluation of all the results of the two experimental series by the analysis of variance technique showed that the observed changes of the synthesis of soluble proteins and albuminoid in rats on restricted diet and of albuminoid after thyroxine injections were significant with confidence limits better than 95 percent in the F test. Changes in the synthesis of soluble proteins in thyroxine rats, however, were not significant.*

Comments

Our experiments clearly show that the synthesis of albuminoid in rat lenses is influenced in opposite directions by restricted diet and by thyroxine, as illustrated by Figure 1.

The decrease in albuminoid formation under restricted diet cannot be, furthermore, due to a decrease in the concentration of soluble proteins. This concentration maximally amounts to only about seven percent at the end of the experimental period, whereas the albuminoid synthesis appears inhibited by 10 to 30 percent. The degree of inhibition of the synthesis of soluble pro-

* We are greatly indebted to Dr. John W. Fertig of the Division of Biostatistics of the Department of Public Health, Columbia University, and also Mr. I. Pompe and Mrs. H. Perlstein of the same division for the statistical evaluation of our data.

TABLE 1

EFFECT OF DEFICIENT DIET ON THE FORMATION OF ALBUMINOID IN LENSES OF RATS

Exp. No.	Animals	Age at Time 0 in Days	No. of Lenses	Experimental Period in Days	Body Weight in gm. at Time		Lens Weight in mg. at Time		Soluble Proteins mg./Lens at Time		Decrease in Synthesis in %	Albuminoid mg./Lens at Time		Decrease in Albuminoid Synthesis in %
					0	t	0	t	0	t		0	t	
I†	Controls	28		60	79	303	16.7	33.1	5.64	10.65		0.65	3.85	
	a		23							9.49			3.81	
	b		23											
	Experimental	28												
	a		23			115		31.2		9.89	15.4		3.32	15.8
	b		23							9.8	3.0		3.33	15.8
II†	Controls	28		44	95		18.2		6.50			1.22		
	a		19			293		29.0		11.10			3.42	
	b		19			293		29.1		11.10			3.43	
	Experimental	28												
	a		19			96		29.4		10.41	16.4		3.02	18.0
	b		19			96		29.4		10.31	17.8		3.01	18.0
III*	Controls	28			74		15.3		5.31			0.90		
	a		18			245		31.8		10.1			3.36	
	b		18	49		245		31.8		10.1			3.38	
	Experimental	28												
	a		18			130		30.1		9.67	8.9		3.16	8.2
	b		18			130		30.1		9.58	10.4		3.13	9.9
IV†	Control	35		20	108		22.0		7.47			1.28		
	a		20			190		25.9		9.13			2.03	
	b		20			190		27.8		9.10			2.00	
	Experimental	35												
	a		20			106		26.1		9.03	6.0		1.94	12.0
	b		20			106		27.5		9.16	−10.0		1.92	11.1
V†	Control	35		20	111		20.4		6.77			1.11		
	a		16			198		28.0		9.13			2.61	
	b		16			198		27.8		9.10			2.63	
	Experimental	35												
	a		16			104		26.8		8.77	15.0		2.33	18.5
	b	—	16			104		27.1		8.98	5.0		2.21	27.7

* McCay diet.
† Pellet diet.

TABLE 2

EFFECT OF THYROXINE ON THE FORMATION OF ALBUMINOID IN RATS

(0.5 mg. L-Thyroxine/100 g. Body Weight [B.W.])

Exp. no.	Animals	Age at Time 0 in Days	No. of Lenses	Experimental Period in Days	Body Weight at Time		Lens Weight in mg. at Time		Soluble Proteins mg./Lens at Time		Decrease in Synthesis in %	Albuminoid mg./Lens at Time		Increase in Synthesis in %
					0	t	0	t	0	t		0	t	
I	Control	35		18	119	189	20.5		6.65			1.03		
	a		15					25.9		8.95			1.90	
	b		15					25.8		8.92			1.92	
	Experimental					137								
	a		15					24.5		8.31	27.2		2.18	32.2
	b		15					24.4		8.21	31.5		2.20	28.0
II	Control	35		18	128	189	19.8	25.8	6.86			1.39		
	a		14					25.6		8.42			2.17	
	b		14							8.47			2.15	
	Experimental					142		24.5						
	a		14							7.80	41.1		2.45	36.0
	b		14					24.5		7.80	41.1		2.30	19.8
III	Control	35		18	81	156	18.4		5.76			0.82		
	a		15					23.7		7.62			1.59	
	b		15					23.8		7.69			1.58	
	Experimental					144								
	a		10					23.7		7.56	6.0		2.00	53.2
	b		10					23.8		7.55	14.7		2.02	57.8
IV	Control	35		10	111	144	21.5		6.19			1.50		
	a		11					24.1		7.61			2.00	
	b		11					28.7		7.59			2.00	
	Experimental					113								
	a		11					24.5		7.59	0.7		2.14	28.0
	b		11					24.0		7.51	6.5		2.15	30.0
V	Control	35		10	78		19.3		6.08			0.87		
	a		13			106		20.2		6.66			1.22	
	b		13			106		20.7		6.66			1.24	
	Experimental													
	a		14			91		18.8		6.32	51.0		1.28	17.1
	b		14			91		19.5		6.66	0		1.29	14.0
VI	Control	35		10	78		15.8		5.49			0.69		
	a		15			103		19.7		6.58			1.10	
	b		15			103		20.0		6.48			1.08	
	Experimental													
	a		15			90		20.9		6.93	31.5		1.22	29.3
	b		15			90		20.7		6.86	38.0		1.19	28.2

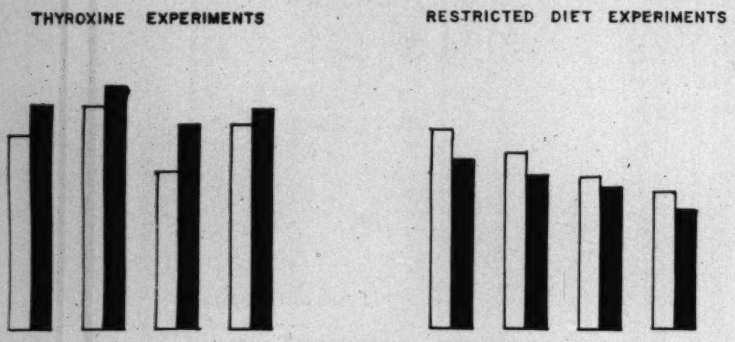

Fig. 1 (Dische and Zelmenis). Effect of restricted diet and thyroxine on albuminoid formation. The four rectangles on the left side correspond to the first four experiments in Table 2; the four rectangles on the right side to the first four experiments in Table 1.

teins and albuminoid is very similar in both cases, and this suggests that the inhibition of the synthesis of both these proteins represents an integrated inhibition of the growth of the whole lens. As a result of this reduced growth, the lens retains to a certain degree the characteristics of a younger lens than corresponds to the chronological age of the rat, and in this sense this inhibition can be considered as a retardation of the aging of the lens.

In considering the results of experiments with thyroxine, it must be pointed out that the effect of thyroxine injections is not due to the continuous injections as such, as in other experiments with other hormones no such effects on the synthesis of lens proteins were observed. Injections of various preparations of ACTH, for instance, in form of cortrophin zinc (Organon) carried out on 8 groups of animals for periods of 10 and 20 days failed to reveal any significant effect on the content of either soluble or insoluble proteins of the rat lenses.

In considering the mechanisms involved in the effect of thyroxine on synthesis of lens proteins, two possibilities come to mind. The simplest explanation is related to the fact that thyroxine is a strongly catabolic hormone which accelerates the breakdown of body constituents by increasing oxidative processes. As albuminoid was shown to differ from soluble proteins by its appreciable content in cystine, it is probable that its formation is due to a transformation of soluble proteins in which an oxidation process plays an essential role. This increase in oxidation processes and in the breakdown of soluble proteins could, therefore, explain the increase of albuminoid and possibly a decrease in soluble proteins found in lenses of rats treated with thyroxine.

In addition to this local effect of thyroxine on the lens metabolism, however, thyroxine could influence the rate of protein synthesis in the lens in another more indirect way by its general systemic action. If the interrelation between the general metabolic turnover in the body and the life span of the animal as illustrated by Rubner's rule has any validity, then the acceleration of the metabolic turnover of the body by thyroxine can be regarded as equivalent to an acceleration of the over all aging of the body.

Although such an effect might be the result of the sum total of local effects of the hormone on all body tissues, it may represent a distinct regulatory systemic mechanism of higher order due to specific interactions between products of the increased metabolism of various tissues, or certain particular tissues. Synthesis of lens proteins could be influenced by this general regulatory mechanism as well as by local hormonal effects of thyroxine.

Now it is clear that, as far as the synthesis

of albuminoid is concerned, the purely local and the systemic influences will exert their action in an identical sense and we may expect a summation of their synergic effects. As far as the synthesis of soluble proteins is concerned, on the other hand, the effects of these two factors will tend to induce changes in opposite directions. The local metabolic effect will tend to decrease the concentration of soluble lens proteins in the lens, whereas the acceleration of the aging process, will tend to increase this concentration. The net result of this competition will depend upon the predominance of one or the other of these influences.

It is noteworthy from this point of view that in experiment III of Table 2 in which the increase in albuminoid synthesis under the influence of thyroxine was more marked than in any other of the six experiments, the decrease in the synthesis of soluble proteins did not materialize. Such a result was to be expected if the systemic influences were particularly pronounced in this case.

Summary

1. Restricted food intake, which leads to a sharp inhibition of body growth in four and five-week-old rats, decreases significantly the rate of albuminoid formation and synthesis of soluble proteins in the lens.

2. In rats injected with 0.5 mg. L-thyroxine per 100 gm. body weight show a significant increase in the albuminoid formation which is accompanied by a nonsignificant decrease in synthesis of soluble proteins.

3. The possible relation of this phenomenon to changes in the rate of aging is discussed.

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Discussion

Dr. V. Everett Kinsey (Detroit): May I suggest a rather obvious experiment, which no doubt Dr. Dische has already considered and that is to do similar experiments using dinitrophenol. This compound is known to increase, and in this case uncouple, some of the metabolic sources of energy.

One point is not entirely clear to me. I do not understand why one would expect that the systemic effect of the thyroxin would do anything but increase the amount of soluble protein.

Dr. Zacharias Dische (closing): I think the systemic effect would tend to increase even the content in soluble proteins. This of course is not known; but if there is any connection between the rate of the basic metabolism and the age, in the sense that an increased rate of the basic metabolism shortens the life of the animal, then we would expect that an increase in the basic metabolism would tend to produce the protein pattern of the lens which corresponds to a later age, and then we would of course expect a higher content of soluble proteins, because older lenses contain more soluble proteins than younger lenses.

Of course, the specific local catabolic effect of thyroxin would tend to decrease it. The hypothetical systemic effect and the well-known catabolic effect of thyroxin act against each other as far as the soluble proteins are concerned, or can be assumed to act against each other.

In the case of albuminoid, however, this is not the case, because if thyroxin has a tendency to increase the rate of oxidation, and we have to assume an oxidative phase in the formation of albuminoid, we can assume that the local effect of thyroxin would tend to increase the formation of albuminoid. The same is true of the hypothetical systemic effect. Therefore, they both are synergic in this case.

THE ACCUMULATION OF C^{14} FROM UNIFORMLY LABELED GLUCOSE BY THE NORMAL AND DIABETIC RABBIT LENS*

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It is now felt that glucose transport into the lens is metabolically mediated, at least in part, and is not dependent solely on simple diffusion. This conclusion is based largely on the following observations. First, when bathed in solutions containing a high glucose concentration and such metabolic poisons as iodoacetate, fluoride or dinitrophenol, the accumulation of glucose within the lens is reduced, suggesting that these substances inhibit some mechanism responsible for the active transport of glucose.[1, 2] Second, when the integrity of the lens surface is disrupted, as by decapsulation, glycolytic activity is reduced.[3] Third, glucose moves across the lens capsule more rapidly than acetate, a metabolite of smaller size.[4]

The opposite effect, that is, an enhancement of glucose movement or utilization has been observed following the addition of insulin to in vitro preparations of muscle,[5, 6] liver,[7, 8] and adipose tissue.[9] Since one of the postulated actions of insulin is to accelerate the active transport of glucose into the cell, or to increase cellular permeability to glucose, [10–12] a similar influence on the lens might be anticipated. It had been observed that insulin did not alter the accumulation or uptake of glucose by normal intact rabbit lenses,[1] but the effect of insulin on glucose accumulation in diabetic lenses remained in doubt due to the initial high and variable levels of lenticular glucose. Although the usual procedures for measuring glucose transport are not adaptable to the diabetic lens, it seemed likely that the accumulation of C^{14} from uniformly labeled glucose might provide a solution to this problem and our attention was thus turned in this direction. Also, the effect of decapsulation upon glucose utilization by the lens was studied by measuring glucose depletion from the surrounding medium. Furthermore, the influence of 1-phenylethylbiguanide (DBI)* an oral hypoglycemic agent, on glucose uptake by the intact rabbit lens was compared with that of insulin.

METHODS

Rabbit lenses were used throughout and were excised by a posterior approach in the manner previously described.[13] To measure the accumulation of labeled carbon from uniformly labeled glucose, the following procedures were then employed. The intact lenses were placed, posterior surface down, in a 50 ml. flask containing 15 ml. of a modified Tyrode's solution[13] having a glucose concentration of either 90 or 200 mg. percent. One microcurie of uniformly labeled glucose was added to this solution. In appropriate instances 0.1 unit crystalline insulin per ml. of media was added. The pH of the medium was adjusted to the physiologic range (pH 7.32-7.48).

The incubation container was flushed with a mixture of 95 percent oxygen and five percent carbon dioxide, tightly stoppered, and

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† Recipient of a Fight-for-Sight Summer student Fellowship Award of the National Council to Combat Blindness, Inc., Summer, 1958.

* Supplied through the courtesy of U. S. Vitamin Corporation, 26 Vark Street, Yonkers 1, New York.

placed in a water bath at 37°C. for a known length of time.

At the end of the incubation period, the lens was removed from the medium, rinsed with medium containing no added isotope, weighed, frozen rapidly and divided equatorially to separate the anterior and posterior segments. In some instances, the complete lens substance was macerated evenly over a one inch aluminum planchet. In other cases, the anterior and posterior lens substances were separated at the time of equatorial sectioning and spread evenly on separate planchets. Similarly, the anterior and posterior capsules were combined on the same planchet in some cases and placed on separate planchets in others. All samples were dried for one hour at 105°C., then assayed for radioactivity using a Tracerlab thin end-window Geiger-Mueller tube attached to a Berkeley Model 2020 scaler.

The fraction of the total activity accumulated in the lens which could be attributed to unmodified glucose was determined by paper chromatography. For these studies, the lens was incubated in the solution with added isotopic glucose as outlined above. At the end of the incubation period the lens was rinsed, weighed, macerated, and the proteins were precipitated with an ether-alcohol mixture. The supernatant fluid and three washings of the precipitate were combined, then evaporated to dryness over a steam bath and the residue was extracted with pyridine at 100°C. for 10 minutes. The supernatant from the latter procedure was then evaporated to dryness in vacuo and the final product was dissolved in water and applied to Whatman No. 1 filter paper for paper chromatographic studies. Carrier glucose was added at the origin to provide sufficient glucose to give a well-defined spot.

Isopropyl alcohol, pyridine, water and glacial acetic acid in the proportions of 8:8:4:1 were employed as the solvent for descending chromatography lasting 16 hours at room temperature. The chromatograms were stained with a solution of 0.5 gm. benzidine, 80 ml. absolute alcohol and 200 ml. glacial acetic acid to outline the glucose spot. The entire strip from the origin to the solvent front was divided into 2.5 by 2.5 cm. sections which were individually assayed for activity under the Geiger-Mueller tube. The insoluble fraction was dissolved in 10 percent KOH, air dried on similar sections of filter paper and counted in the same manner as the sections of the chromatographic strips. Results are expressed as percentage of the total counts present under the area corresponding to the stainable glucose spot.

In studies measuring glucose depletion from the bathing fluid, the lens was excised as before, then the following procedures were employed. If decapsulated lenses were to be studied, the capsule was removed with a pair of small forceps and both the decapsulated lens and its capsule were placed together in 3.0 ml. of the previously listed medium containing 90 mg. percent glucose. Crystalline insulin (0.1 unit per ml.) or DBI (0.1 mg. per ml.) was added to appropriate tubes. The tube was flushed with 95 percent oxygen and five percent carbon dioxide gas mixture and incubated for six hours at 37°C. After incubation, the lens was removed from the tube, rinsed, blotted dry and weighed. Decapsulated lenses were assumed to have the same weights as their intact mates.

For glucose analysis, an aliquot of the medium was precipitated with barium hydroxide and zinc sulfate, and the glucose concentration determined by the method of Somogyi.[14] Glucose depletion was calculated by subtracting the glucose content of the experimental solution from that of a control handled in the same manner but without the addition of a lens. It is expressed as milligrams of glucose disappearing per gm. of wet lens per hour of incubation time.

Animals were made diabetic by the intravenous administration of five percent alloxan solution in a dosage of 175 mg. per kilogram body weight. After four days, the

level of the blood sugar was determined and those rabbits having a blood glucose level above 400 mg. percent were used for lens studies.

Results

A. C^{14} accumulation studies

As the study of the accumulation of isotopic carbon from uniformly labeled glucose by the lens progressed, it became apparent that, under identical conditions, the weight (age) of the lens significantly influenced the amount of activity recorded. Apart from the variations noted in individual lenses, it was found that, as the lens weight increased, the recorded accumulation of activity per lens decreased in a linear fashion (fig. 1). Several factors may account for this observation:

First, with increasing lens age, there is a corresponding decrease in metabolic activity.

Second, as the lens grows, the volume to surface ratio increases.

Third, as the mass of lens substance increases, self-absorption of the radioactivity is greater and the counting technique becomes less reliable for determining total activity. The decreasing activity recorded when lens weight increases makes it impossible to compare directly one experimental situation with another in terms of absolute counts per minute per lens when the weights of the lenses in the two situations differ to any extent. However, by multiplying counts per minute per lens by the lens weight a constant relationship was obtained. This permits one to compare lenses of different weights directly (fig. 1). This relationship was observed whether lenses were from diabetic or normal animals.

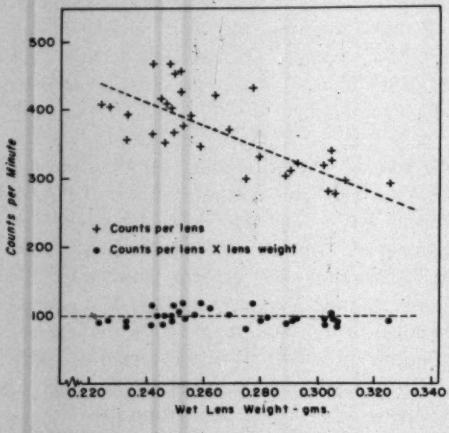

Fig. 1 (Giles and Harris). Activity accumulated by normal lenses after being incubated at 37°C. for six hours in 15 ml. of modified Tyrode's solution containing either 90 or 200 mg. percent glucose and one μc. C^{14} uniformly labeled glucose.

The spread in counts per minute per lens noted at any one lens weight was in large extent due to variation between different rabbits. Lenses from the same animal, run under identical conditions, showed good agreement in activity accumulated. Here, as in all lens studies, the two lenses from the same animal should ideally make up the complete experiment. Obviously under certain conditions groups of animals must be compared with each other, as the comparison of normals with diabetics. For this reason the previously mentioned empirical step of multiplying lens weight by counts per minute per lens was undertaken to allow different groups of animals to be directly compared.

One of the first problems was to determine if the time course of C^{14} accumulation closely enough approximated the time course of the accumulation of fermentable reducing substance previously reported[1] in order that C^{14} accumulation from a medium containing uniformly labeled glucose may be taken as another index of glucose transport across the lens surface. Normal rabbit lenses were incubated in a medium containing uniformly labeled glucose for various time intervals to determine the kinetics of the accumulation of C^{14} by the lens substance (fig. 2).

The rate of accumulation of the labeled carbon becomes progressively slower until by 24 hours it is essentially at a steady state. This curve closely follows the one previously obtained when the accumulation of reducing substances by the lens was measured. We

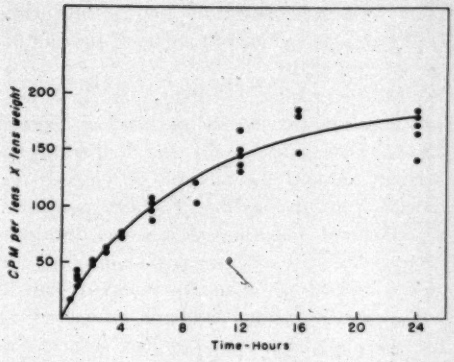

Fig. 2 (Giles and Harris). Time course of activity accumulated by normal lenses after being incubated at 37°C. for various time periods in 15 ml. of modified Tyrode's solution containing 200 mg. percent glucose and one μc. C^{14} uniformly labeled glucose.

have accordingly considered that C^{14} accumulation under conditions given above as an index of glucose transport into the lens. After six and 12 hours' incubation, the accumulation of activity from a medium containing 200 mg. percent glucose was the same as that from one containing 90 mg. percent glucose (the same amount of labeled glucose being present). Thus the concentration of unlabeled glucose did not influence the uptake of the label. The technique is thus applicable to the diabetic lens when a high glucose concentration is encountered.

The amount of isotope accumulated in the lens capsule is shown in Figure 3. Per unit weight, the activity accumulated by the capsule was considerably greater than that accumulating in the lens substance itself. This would be expected from the spatial relationship of the capsule to the medium as compared to the remainder of the lens. A higher metabolic activity near the surface may also be a factor. When the anterior and posterior capsules were counted separately, generally 50 to 100 percent more activity was found in the anterior capsule. Similarly the anterior portion of the lens substance contained a significantly higher level of activity than did the posterior segment.

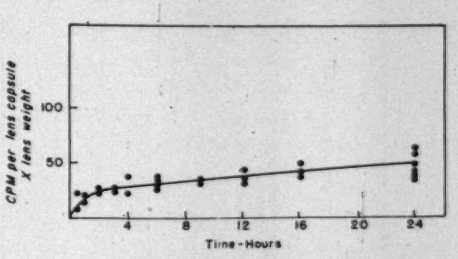

Fig. 3 (Giles and Harris). Time course of activity accumulated by normal lens capsules after being incubated at 37°C. for various time periods in 15 ml. of modified Tyrode's solution containing 200 mg. percent glucose and one μc. C^{14} uniformly labeled glucose.

Paired lens systems were used to measure the effect of insulin on the accumulation of C^{14} by both normal and diabetic intact lenses. No significant effect of insulin was noted on the uptake of activity by the normal lens (fig. 4), nor was any change seen on the addition of insulin to diabetic lenses. Using the function of lens weight times counts accumulated, the diabetic lenses showed a slightly higher level of activity than did the normal lenses (fig. 5) and this difference was statistically significant.

To estimate the percentage of the ac-

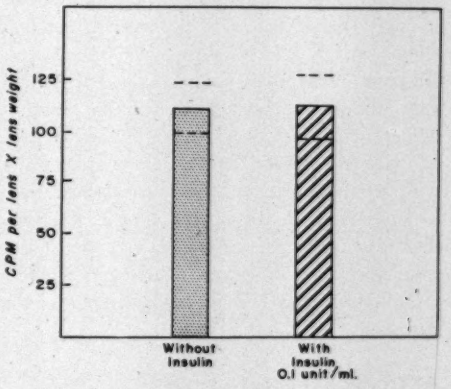

Fig. 4 (Giles and Harris). Effect of insulin on C^{14} accumulation of paired normal lens systems incubated at 37°C. for six hours in 15 ml. of modified Tyrode's solution containing 200 mg. percent glucose and one μc. C^{14} uniformly labeled glucose.

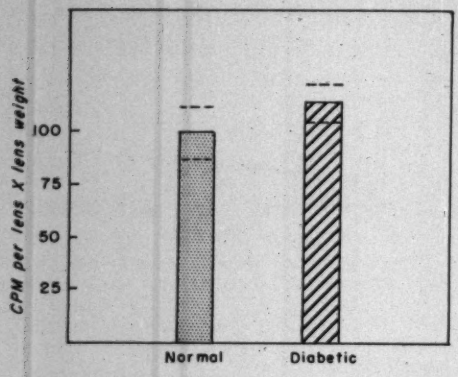

Fig. 5 (Giles and Harris). Comparison of C^{14} accumulation of normal and diabetic lenses incubated at 37°C. for six hours in 15 ml. of modified Tyrode's solution containing 200 mg. percent glucose and one μc. C^{14} uniformly labeled glucose.

cumulated activity in the lens contributed by unmetabolized glucose, paper chromatographic separation of the lenticular monosaccharides was undertaken. That activity of the lens which migrated a distance corresponding to a stainable glucose spot was measured and compared to the total activity found in the lens. In the normal lens, about 62 percent of the total activity migrated this distance. In the diabetic lens, 84 percent of the total activity migrated this distance. Following the extraction procedure outlined above, nearly all the activity on the chromatographic strip was found under the glucose spot, the remainder of the activity being in the ether-alcohol precipitate. When extracts of normal lenses were chromatographed without the addition of carrier glucose, only a suggestion of a stainable spot at the same distance from the origin as a control glucose spot was observed. However, diabetic lenses showed a heavily stained spot at this distance.

B. Glucose depletion studies

The normal intact lens depletes glucose from the medium at a rate of slightly over 0.7 mg. per gm. of wet lens per hour of incubation time when incubated for six hours. Upon removal of the capsule, this depletion was reduced to about 55 percent of the normal value.

Glucose uptake by intact diabetic lenses was considerably lower than that of normal lenses. Decapsulation of the diabetic lens further reduced the amount of glucose removed from the medium. In fact, in several decapsulated diabetic lens systems, the glucose level of the medium was slightly higher at the end of the incubation period than it was at the beginning. It seems probable that these findings are in large part due to the initial high level of glucose in diabetic as compared to normal lenses. In a series of rabbits having blood sugar levels ranging from 360 to more than 960 mg. percent at the time of death, an excellent correlation was noted between the decreasing uptake of glucose by both intact and decapsulated lenses and the increasing blood sugar level of the animals.

As had been previously noted,[1] the addition of insulin to the intact lens system caused no increase in glucose uptake. However, the addition of 0.1 unit of insulin per ml. to a decapsulated lens system increased glucose disappearance by about 28 percent in comparison with its untreated mate.

In contrast to the failure of insulin to affect the intact lens in vitro, the addition of

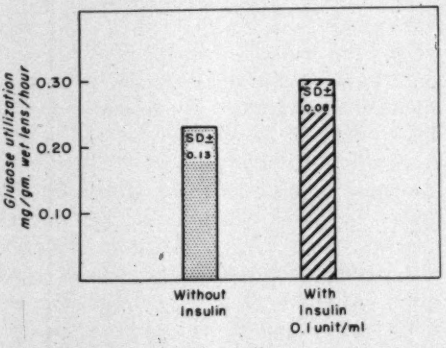

Fig. 6 (Giles and Harris). Comparison of glucose depletion by paired decapsulated lenses with and without added insulin incubated at 37°C. for six hours in 3.0 ml. modified Tyrode's solution containing 90 mg. percent glucose.

TABLE 1

GLUCOSE

Depletion by paired normal lens systems with and without added DBI (0.1 mg. per ml.) incubated at 37°C. for six hours in 3.0 ml. modified Tyrode's solution containing 90 mg. percent glucose. Results are expressed as mg. glucose depleted per hour per gm. wet lens weight.

Lens Pair No.	With DBI	Without DBI	Difference
1	1.008	0.780	0.228
2	1.200	0.720	0.480
3	1.200	0.708	0.492
4	1.224	0.744	0.480
5	1.296	0.732	0.564
6	1.152	0.696	0.456
7	1.296	0.660	0.636
8	1.140	0.624	0.516
Av.	1.189	0.708	0.481

0.1 mg. DBI per ml. to the medium caused a dramatic increase in the glucose uptake of normal intact lenses. Eight normal lenses not treated with DBI showed an average uptake of 0.708 mg. glucose per gram of wet lens per hour while the paired lenses exposed to DBI exhibited an average uptake of 1.189 mg. glucose (table 1).

DISCUSSION

If the lens behaved like many other tissues, it might be expected that the addition of insulin to the in vitro system would result in an increased movement of glucose into the lens. However, no effect of insulin on the accumulation of either fermentable reducing substances or C^{14} from uniformly labeled glucose by the intact lens has been noted. In addition, diabetic lenses showed no increase in accumulation of the isotope when insulin was added in vitro, although other tissue preparations from diabetic animals generally show a good response to insulin under these conditions.

These observations on the lack of an effect of insulin on the lens in vitro have been strengthened by recent studies utilizing intact normal and diabetic rat lenses. Farkas and Patterson[15] reported the absence of an insulin effect on glucose depletion by such isolated lenses.

The lens thus resembles the erythrocyte into which glucose moves at a more rapid rate than would be predicted from its molecular size. Insulin does not influence this movement.[16]

This failure of insulin to affect the intact lens in vitro may be due to one or more factors:

First, insulin may not enter the aqueous humor in physiologically significant quantities and the potential for its use by the lens therefore has not been developed.

Secondly, insulin as presented to the lens in the artificial in vitro preparation may not be the active principal normally needed by the lens, that is, some alteration of its structure may be necessary before it becomes active.

Third, a cofactor not present in the lens itself but normally supplied by other tissues may be necessary before insulin can act on lenticular metabolism.

Fourth, insulin although present in the aqueous humor and the bathing medium, may be prevented from acting on the lens because an impermeable capsule does not permit penetration of the hormone.

Farkas and Patterson further reported that pretreatment of rats with insulin prior to isolation of the lens resulted in an increased glucose uptake by both normal or diabetic lenses. (It has also been shown that pretreatment of rats with insulin results in a marked increase in glycogen formation by liver slices, whereas the addition of insulin to the medium was ineffective.[17]) To further compound the question of the effect of insulin on the lens, Farkas and Patterson found that this facilitory effect of pretreatment could be almost abolished by removing the liver. This observation suggests at least three possible mechanisms of action:

First, the liver may produce a cofactor that, in conjunction with the insulin molecule, causes an increased glucose uptake.

Second, the liver may alter the insulin molecule to some form capable of acting upon the lens.

Third, the stimulation of the administered

insulin may result in the formation by the liver of an unknown substance which acts on the lens.

The measurement of insulin levels in the aqueous humor gives at least a partial answer to the question of which of these mechanisms may be operative. The level of activity which can be considered biologically active insulin (as we know it for other tissues) which enters the aqueous has been shown to be considerably lower than the plasma levels following the intravenous injection of radioiodinated insulin.[18] It is considered that insulin does not enter the aqueous in physiologically significant quantities and the tissues bathed by this fluid are in a state of relative diabetes at all times (using the insulin levels normally available to other tissues as a baseline). However, the possibility that much lower levels of insulin will suffice for normal function in this tissue must not be ignored.

The observation that a substance identifiable as insulin by paper electrophoresis was not present in significant quantities in the aqueous seems to rule out the possibility that the effect of the liver is to supply a cofactor which combines with insulin to act upon the lens. If the liver modifies the insulin molecule in some fashion, it must do so to such an extent that it no longer has the electrophoretic characteristics of the parent structure. The suggestion that pretreatment stimulates the production of an active factor by the liver is a question not yet answered. The species specificity of this pretreatment effect must also be considered.

The disruption of lenticular integrity by decapsulation results in marked changes in the metabolic pattern of the lens. Although oxygen consumption by the lens increases with decapsulation,[19] other major functions decrease following this maneuver. For example, glucose uptake decreases to about 55 percent of its normal level. Other evidences of impaired metabolic activity include decreased production of lactic acid[20] and decreased accumulation of labeled phosphorus[21] by the lens substance. The presence of the lens capsule is thus essential for the maintenance of proper cell life in vitro. In vivo, rupture of the capsule results in a progressive absorption of the lens substance by the aqueous humor—a process undoubtedly similar in part to the progressive disintegration of the decapsulated lens seen in in vitro preparations. This lessening of activity when the capsule is removed must in large part be due to a destruction of cellular integrity.

An earlier report[22] indicated that the presence of insulin in the incubation fluid increased glucose uptake by the decapsulated lens to 350 percent of its control level. Another report[1] of insulin action on a disrupted lens system showed a smaller but significant effect of insulin on glucose uptake in lenses with capsular integrity destroyed by an incision through the capsule. The finding of about a 28 percent increase in glucose uptake in decapsulated lenses upon the addition of insulin more closely corresponds to the degree of increase found in the study employing lenses having the capsule nicked than in the former study of decapsulated lenses.

Why this in vitro action of insulin upon glucose uptake by the lens is seen when capsular integrity is destroyed is not known. Possibly the capsule constitutes an absolute barrier to the action of insulin, preventing it from moving to the cells beneath, but with the removal of the barrier, insulin becomes effective. Rupture of the lens fibers, either during decapsulation or during the incubation period, may allow insulin to act on intracellular units now released to the fluid. Williams, et al. have shown that insulin distributes itself in a characteristic intracellular pattern following intravenous injection into rats.[23] However, this again is an in vivo situation with intact cells in contrast to the situation obtaining with a decapsulated lens.

Many examples of the depressant effect of diabetes on glucose utilization by various tissues may be found in the literature.[24, 25, 26] In lenses from diabetic animals, glucose up-

take is markedly reduced. However, as mentioned above, this reduction may in large part be due to the high level of glucose in the lens itself at the beginning of the incubation period. This simple explanation for the reduced glucose uptake of the diabetic lens does not explain why the isotopic carbon accumulation from uniformly labeled glucose by these tissues is not depressed. The fact that a greater proportion of the radioactivity found in the diabetic lens appears as unaltered glucose may again be due to the initial high level of unlabeled glucose available for the metabolic needs of the lens which in turn could exert a "sparing effect" on the labeled glucose moving into the lens.

Since one of the suggested modes of action of DBI is the stimulation of anaerobic glycolysis and since the major part of the energy required for lenticular function can be derived from the utilization of glucose via this pathway,[27,28] it is not surprising to find that DBI stimulates glucose uptake. However, the action of DBI on the glucose metabolism of the lens requires much more extensive exploration.

Summary

1. Recorded C^{14} accumulation by lenses decreased as lens weight increased.

2. Insulin had no effect on C^{14} accumulation from uniformly labeled glucose by either normal or diabetic intact lenses.

3. The anterior portion of both the lens capsule and lens substance accumulated more activity than did the corresponding posterior portions.

4. Approximately 62 percent of the accumulated activity in normal lenses and 84 percent in diabetic lenses migrated to a point corresponding to a stainable glucose spot on paper chromatography.

5. Decapsulation resulted in a lowered glucose uptake by both normal and diabetic lenses. Diabetic lenses (intact or decapsulated) took up less glucose from the medium than did normal lenses.

6. Addition of insulin to a decapsulated lens system increased its glucose uptake.

7. DBI caused a marked increase in the glucose uptake of normal intact lenses.

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Discussion

Dr. John W. Patterson (Nashville, Tennessee): We know that cataracts develop in insulin deficiency diabetes, whether this is the juvenile diabetes in man or experimental diabetes in animals. We know, further, that these cataracts can be prevented by adequate insulin therapy.

The beneficial action of insulin might be due to a direct effect on the lens or to an indirect effect on the metabolism of some other organ. The preponderance of evidence supports the latter concept.

However, certain observations have tended to confuse the issue. Drs. Giles and Harris are to be congratulated for obtaining and reporting additional data which help to clarify the problem.

The observation by Ross, of an increase in the disappearance of glucose following the addition of insulin to the media in which decapsulated rabbit lenses were incubated, has been interpreted by many workers as proof that insulin acts directly on the lens. The present paper confirms the qualitative findings of Ross for decapsulated lenses, but it also reemphasizes the fact that insulin does not directly affect the uptake of glucose by the isolated but intact lens.

Therefore, from the standpoint of physiology it is probably correct to assume that insulin does not act directly on the lens, and that observations made with decapsulated lenses are interesting but of doubtful value in elucidating the role of insulin in the whole animal.

A few years ago we reported that the disappearance of glucose from the media in which the lenses of diabetic rats were incubated was 60-percent less than observed for normal lenses. These findings were justly criticized because they did not take into consideration the level of reducing sugar within the lens. When the disappearance of reducing sugar from the total system of lens plus medium was measured, the lenses from diabetic animals metabolized about one-third less than those of normal animals.

This agreed with similar observations on rabbits made in Dr. Kinsey's laboratory. The present paper shows that glucose enters the lens of a diabetic animal more rapidly than it does a normal lens. The difference is not great, however, and may be accounted for by an increased weight due to hydration which is known to occur in lenses from diabetics.

The present paper also shows that lenses from diabetics do not metabolize intralenticular labeled glucose as rapidly as normal lenses. The amount metabolized after six hours is about 50-percent less in the lenses from diabetics.

Allowing for the fact that this may be exaggerated by the "sparing effect" of the glucose pool within these lenses, it is consistent with the other observations. Therefore, it is probable that the defect in metabolism in lenses from diabetics are not related to permeability of glucose into the lens but to some later step in the metabolism. Furthermore, this is consistent with the concept that insulin does not act directly on the lens.

I would again like to congratulate the authors, and ask the following question: I would like to

know whether they have any data on the effect of DBI on lenses from diabetic animals, and whether they would care to speculate regarding the possible beneficial effects of DBI in preventing cataracts.

DR. KENNETH GILES (closing): Thank you, Dr. Patterson. We haven't yet studied the effect of DBI on diabetic lenses yet. One of the students on our laboratory hopes to continue work on DBI during the coming year. Possibly he will report on his findings next year.

As far as speculating on the effect of DBI in the treatment of cataract is concerned, I don't know. One might expect that if cataract is due to an impairment of energy supply to the lens, and since DBI apparently increases glucose consumption that it may be of some help in cataract prevention.

A CONTRIBUTION TOWARD THE STANDARDIZATION OF CLINICAL ELECTRORETINOGRAPHIC EQUIPMENT*

JERRY HART JACOBSON, M.D., AND GIDON F. GESTRING
New York

The human electroretinogram has been applied to problems of clinical ophthalmology for over 15 years. Since the pioneer work of Riggs and of Karpe first demonstrated the practicality of the basic method, a large number of reports dealing with the electroretinogram findings in avariety of diseases have been published.

One of the problems confronting an investigator in this field is the difficulty in correlating his findings with those obtained in other laboratories. The elimination of this difficulty is one of the aims of the new International Society for Clinical Electroretinography, recently formed, of which I am honored to be a member of the founding committee.

Toward the end of aiding in the standardization, we should like to present some of our recent work dealing with instrumentation.

The amplitude and conformation of the electroretinogram recorded from a particular individual depends upon a variety of factors, including:

A. CHARACTERISTICS OF THE STIMULATING LIGHT

1. Intensity—including such factors as distance from patient.
2. Spectral composition.
3. Duration.
4. Repetition rate.
5. Presence or absence, and spectral compostion of background light.

B. CHARACTERISTICS OF THE RECORDING SYSTEM

1. Type and size and situation of contact lens electrode.
2. Frequency response, time constant, gain, noise level and differential ration of the amplifying system.
3. High frequency response and paper speed of ink writers.
4. Film speed of moving film camera, if used.

C. CHARACTERISTICS OF THE PATIENT

1. State of dark or light adaptation—duration, intensity and spectral composition of preadapting light; level of illumination, if any, during adaptation.
2. Pupil size.
3. Retinal functional integrity—including all of the variables bearing upon this function.
4. Central nervous system activity level.

As can be easily recognized, a conclusion relative to the retinal integrity, since it is dependent upon all the other variables mentioned above, is on tenuous grounds unless the other factors are standardized.

Thus far, each laboratory performing

* Department of Electrophysiology, New York Eye and Ear Infirmary.

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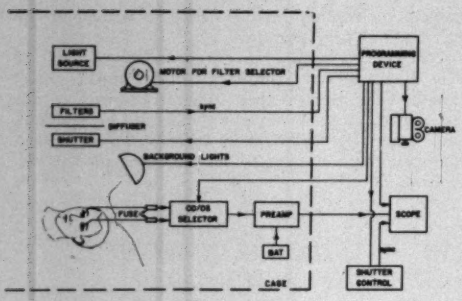


Fig. 1 (Jacobson and Gestring). Block diagram of system.

these studies has solved this problem by establishing for itself, a set of operating conditions, used in all its recordings. As a result, a variation from the normal findings under these conditions, is significant. It is only when attempts are made to compare results obtained in several laboratories that problems due to varying conditions arise. In an attempt to aid toward the standardization of testing conditions, we have devised and built the instrument described below. Our aim was to have an accurate system, relatively inexpensive to construct and maintain, with components easily available and simple to operate.

A block diagram of the unit is shown in Figure 1. The patient is positioned upon a stretcher within a shielded chamber, with a contact lens electrode in each eye. The electrodes lead, by way of two fuses, to the input box of the amplifying system. The need for the fuses is shown in Figures 2 and 3, which demonstrate how tube and/or condenser failure or interruption of the grounding circuit may, in an unfused circuit, cause damage to the eye.

The input box consists of a relay-actuated switching device (fig. 4) to allow choice of right or left eye, alternately. The advantages of this switching device instead of the use of two channels are, economy, since it eliminates the need for multichannel amplifying and recording devices and, more important, assurance of identity of amplifying and re-

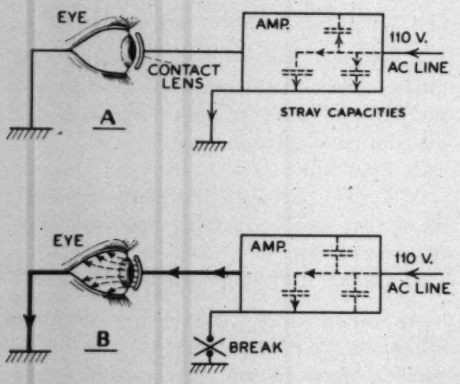


Fig. 2 (Jacobson and Gestring). (A) Normal dissipation of stray capacities by grounding of amplifier (B) Manner in which interruption of ground will cause damage to the eye.

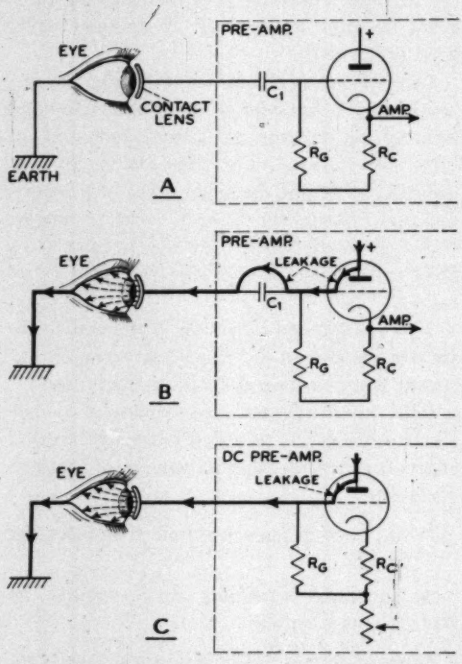


Fig. 3 (Jacobson and Gestring). (A) Normal condenser-coupled input to amplifier. (B) Breakdown of tube and condenser, showing path of damaging current through eye. (C) D-C amplifier, with tube plate to grid (contact), showing path of damaging current.

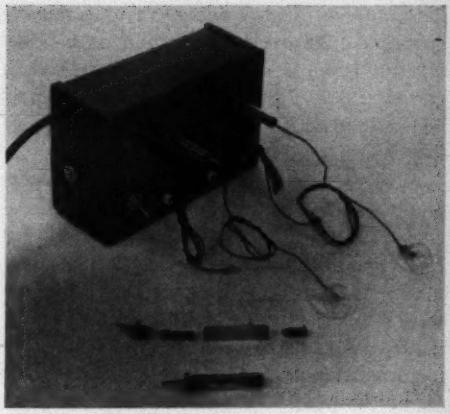


Fig. 4 (Jacobson and Gestring). Input box showing assembled and disassembled fuses in front. Box contains relay for switching from right to left eye.

Fig. 5 (Jacobson and Gestring). Patient in position on stretcher. Input box at head of stretcher, with pre-amplifier on bottom shelf of stretcher.

cording systems, when comparison of the two eyes is important. The relay controlling the selection of eye is driven by a battery, in common with all the equipment within the shield, to eliminate AC artefact.

Since the leads from the contact lenses to the amplifier are at relatively high impedance, it is best to keep them as short as possible, to minimize artifact. To achieve this, the preamplifier and its batteries are mounted beneath the patient on the stretcher (fig. 5).

The last stage of this preamplifier (fig. 6) is a cathode follower output, allowing the use of a shielded low impedance cable to lead to the oscilloscope, which is situated outside of the shielded chamber.

The characteristics of this amplifier, with

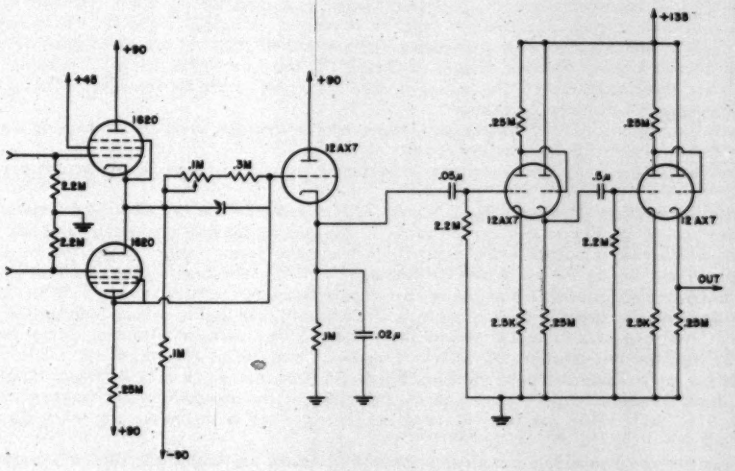


Fig. 6 (Jacobson and Gestring). Circuit of pre-amplifier.

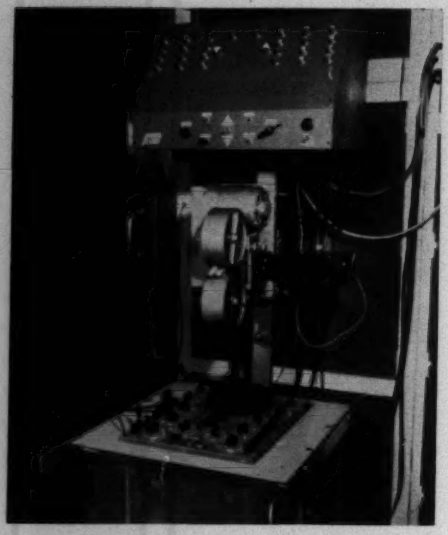


Fig. 7 (Jacobson and Gestring). Oscilloscope, camera and programming device. Data chart assembly to right of camera, for recording patient's name and date. Cables at right lead to the shielded chamber.

the values presented here give us a flat response to 1,000 cycles per second, a time constant of approximately one-half second, a gain of 2,000, a differential rejection ratio of better than 1:50,000, and a noise level of less than 5.0 microvolts peak to peak.

A single beam oscilloscope will serve perfectly well for recording. The use of the oscilloscope is, in our opinion, essential for recording all the details of the electroretinogram. The records obtained with the ink writer of the electroretinogram machine, which has hitherto been in general use, are distorted to a degree by the mechanical inertia of the pens. We feel that many fine details of the record may be thus lost.

Mounted above the oscilloscope and its recording camera (fig. 7) is a programming device. It is our feeling that the use of light stimuli and background illumination of a variety of spectral compositions, or what we have called "Spectro-differential electroretinography," is of great importance.

Fig. 8 (Jacobson and Gestring). Circuit diagram of the programming device (to left of dotted line) and photostimulator and background lights (to right of dotted line).

At the lower left corner is the line feeding through two three ampere fuses into the master on/off switch. When in the off position this switch energizes the battery charger. This charger, situated outside of the shielded chamber where the patient is placed, maintains the charge on the battery supply for the entire apparatus during intermissions and by overnight charging. Only DC is brought into the shielded chamber. Immediately above the charger on the circuit diagram are three delay tubes, the first two of which have a delay of three minutes each and the third of which delays 45 seconds. During the automatic cycle, these tubes control the period of dark adaptation, start the recording camera, turn off the room lights and control the stimulus shutter.

The manual/automatic (M-A) switch just above this allows the investigator to stop the automatic cycle if he wishes to alter the cycle at any point.

At top left is a delay relay which activates a buzzer at the end of the cycle and inactivates the shutter mechanism.

A 10 deck, 25 position stepping relay is shown in the center of the drawing. The activating six volt coil is located below it. The switch can be made to advance by manually pressing the push button (S) below it, in which case it advances one step: by pressing the button "auto," which advances the switch 12 steps per second, or, by the automatic programming device, consisting of one rpm synchronous motor and two microswitches, for advances of one or two steps per minute.

The top deck of the stepping switch controls 26 indicator pilot lights, which indicate the position of the switch at any particular time. The second deck supplies the automatic advancing device between position 1 to 25, and the last position, 26, activates the delay for end of record cut-off. Decks 3 through 9 energize relays controlling the light stimulus filters (3, 4, 5 and 6), and background lights (7, 8, 9). The tenth deck, bottom, energizes a relay in the input box of the preamplifier for right or left eye selection (O.D./O.S. Sel). When the relay is energized the right eye is in the circuit; when the relay is inactive, the left.

To the right of the dotted line are all the components which are within the shielded chamber with the patient: Filter selector wheel, its motor (M), O.D./O.S. switch, background lights (red, green and blue), projector light source, and the diagram of the connecting cable.

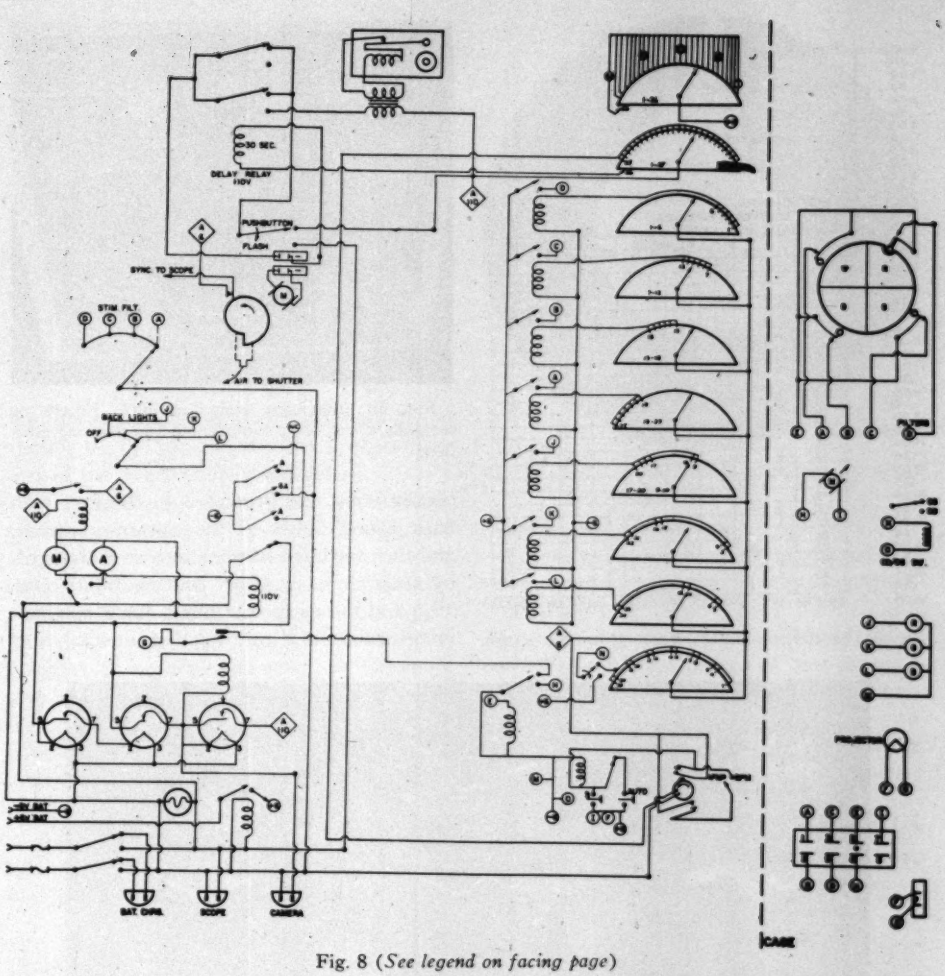


Fig. 8 (*See legend on facing page*)

The programming device, seen at the top of Figure 7, and whose circuit is shown in Figure 8, is essentially an automatic means of controlling the choice of color of stimulus and background light, selection of each of these through a pre-set cycle, controlling duration of dark adaptation and of which eye is being recorded, actuating the shutter mechanism, the sweep of the oscilloscope and the film drive of the camera.

The program we have chosen is shown in Figure 9. This system allows us to record from each eye with each of 13 different stimulus-background combinations, each step repeated 10 times in sequence. Modification of the program may be necessary as time progresses.

The program is controlled by a stepping relay, of the telephone 25/10 type. It allows for a period (in our situation, seven minutes) of dark adaptation. Toward the end of this period the motor of the recording camera is started, and the pump used to drive the stimulus light shutter is activated. An exposure is made of a card bearing the name of the patient and the date. Stimuli of the

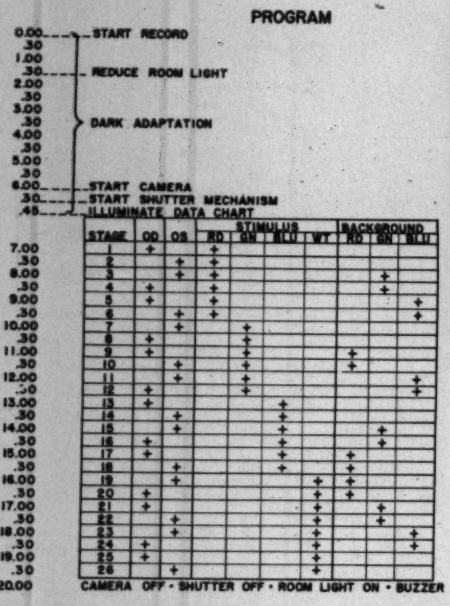


Fig. 9 (Jacobson and Gestring). Program in use.

Fig. 10 (Jacobson and Gestring). Filters on rotating wheel, with microswitch for ensuring position.

chosen color are presented to the eye, with background lights of the chosen sequence, and then the color of the stimulus is changed, by rotation of a wheel bearing filters (fig. 10), and the cycle continued. Each response is recorded on a continuously moving film,

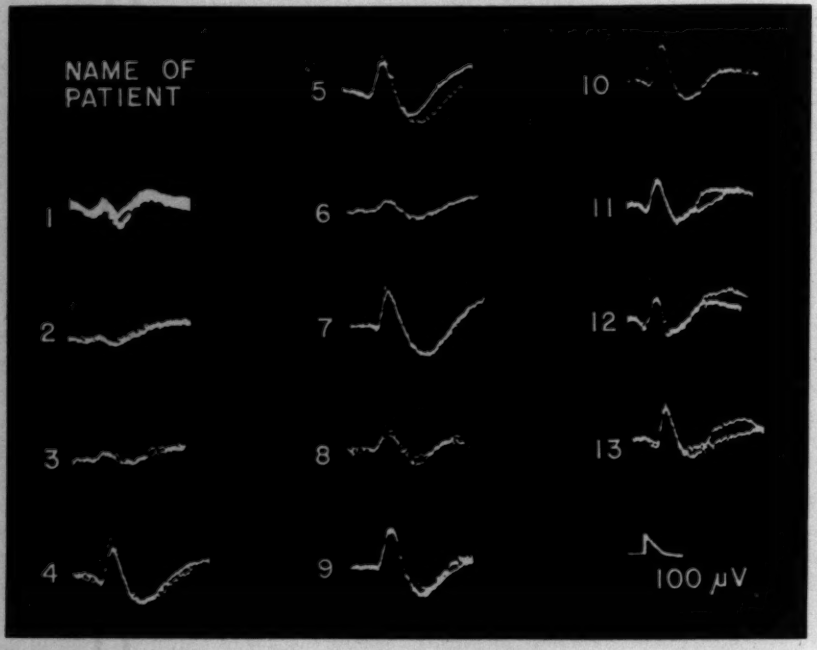


Fig. 11 (Jacobson and Gestring). Superimposition of ten traces in each of 13 recording situations.

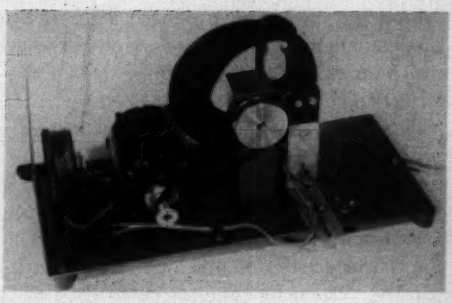


Fig. 12 (Jacobson and Gestring). "Wheel" simulator for production of artifical electroretinogram.

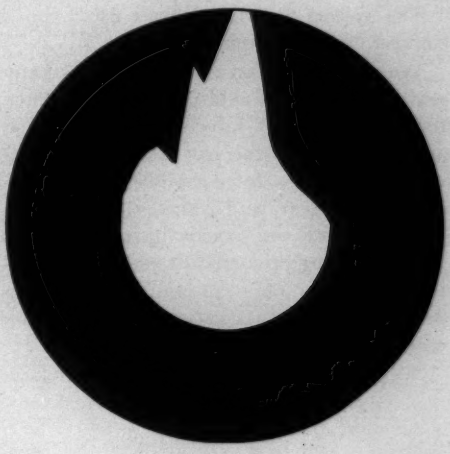


Fig. 13 (Jacobson and Gestring). Pattern for wheel.

the programming device coordinating the shutter of the camera and the synchronization of the sweep of the oscilloscope.

The camera we use (Grass Kymograph camera) is run at 10 cm./min., and uses about six feet per record. Paper may be used instead of film, and can be fastened directly to the patient's chart. If negative film is preferred, and it is by us, to facilitate superimposition of several responses to eliminate artefact, a positive print is readily made and filed, on one 8 by 10 inch sheet of contact paper. Figure 11 shows such a record, in this a superimposition of ten responses to each of 13 situations.

We use incandescent lamps as the stimuli light source, and control the test flashes with a large photographic shutter. Our stimulus duration is approximately 1/20 second.

The question of the most appropriate duration and source of light is under consideration by the International Society for Clinical Electroretinography, and we would rather not specify further details of this aspect at this time. The use of a stroboscopic light source, we feel frequently leads to the production of small artefacts at the time of firing and requires introduction of high-potential into the area of the patient, which may also produce artifact. Also, the spectral composition of the stroboscope may not be best for the work undertaken.

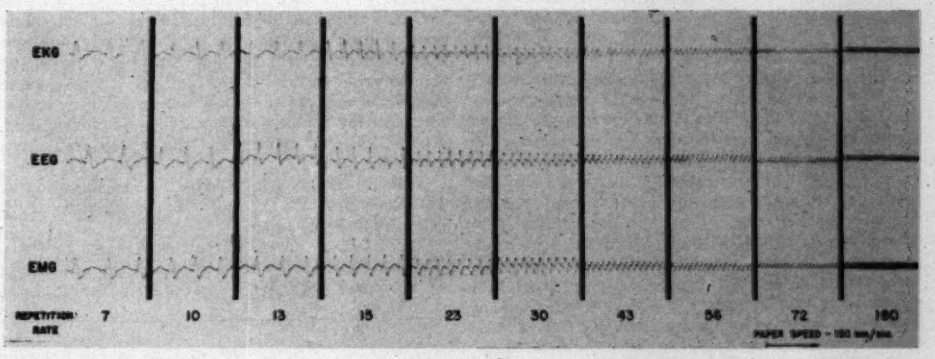


Fig. 14 (Jacobson and Gestring). Output of electroencephalogram amplifiers with device, showing variation due to varying time constants.

The system described allows us to run a complete test in 20 minutes.

We understand that the components of any sequence of stimuli will necessarily interact, the preceding ones affecting the adaptation and response of the eye to the following ones. It is only by establishing some sequence, however, and considering it a constant, that we can practically perform this type of testing on a significant group of patients. It is our aim in presenting this instrument to aid in the standardization of a recording technique.

As a separate device to aid in this standardization we have also produced (fig. 12) a small simulator, essentially a revolving wheel which interrupts the light falling upon a photoelectric cell. The pattern pasted upon the wheel (fig. 13) is made in such a form as to produce a wave form of the "normal" electroretinogram. It is our purpose in doing this to allow laboratories to use this sort of device, which can be easily reproduced, to determine the characteristics of this amplifying and recording system by comparison to the output of a widely used electroencephalogram amplifier (fig. 14).

880 Fifth Avenue (21).

Discussion

G. M. Breinin, M.D. (New York): The authors have pointed out the variable results obtained in clinical electroretinogram testing in different laboratories. They have listed the parameters which influence the electroretinogram. They are indeed to be congratulated for emphasizing this most important aspect of the problem. My only criticism in this connection is that the subject merits an exhaustive treatment rather than mere statement. We shall all be grateful if the authors will present us with such a critique in the future. Of particular importance would be an exposition of the relative weight of these variables upon the configuration of the electroretinogram. This would play a vital role in the establishment of meaningful testing programs.

The authors in their technical exposition have described a thing of some importance in many forms of neurophysiological testing but one which is generally overlooked. I refer to the fusing of the input leads. Although the potential hazards of unfused leads have long been recognized they have received scant consideration. I shall certainly employ such protective measures in my own work. I would appreciate knowing the fuse characteristics and what levels of current are actually dangerous.

The question of ink writers versus oscilloscope in clinical recording definitely may be argued. Armington has pointed out that ink writers provide all the essential data required in clinical records. The existence of electroencephalogram apparatus in many centers and the paucity of oscilloscopes is an argument in favor of ink writers for clinical electroretinogram testing. There would be far fewer workers in the field if electroretinogram recording were restricted to oscilloscope systems. Furthermore, paper recording enables the selection of a stable baseline, a most important consideration. To achieve this with oscilloscopes requires an additional monitoring oscilloscope since triggering of the trace is the technique generally employed.*

It is not at all accepted that stroboscope stimulation is inferior to incandescent lamps. For high intensity stimuli an incandescent lamp of excessively high wattage is required.

I should like to add for consideration that flicker electroretinography, which is not mentioned by the authors, is an important tool in photopic analysis. The electroretintogram simulator is a nice idea for calibrating equipment and may prove helpful.

With respect to automatic programming in general, does it not introduce rigidity into the experimental protocol, tending to hamper rather than further the progress of clinical electroretinography? Perhaps a variety of protocols would introduce a suitable flexibility. The answer to this question will no doubt await further experience. The authors point out the interaction of the program test situations. This seems a serious problem in extracting meaningful data. In their protocol they obtained one trace every three seconds which obviously altered the state of the receptive apparatus.

Finally, I would like to point out that one may seriously question the meaningfulness of automatic programmed systems, one may definitely question the convenience, and one may certainly question the economy. Nevertheless, the authors have struck a significant and praiseworthy note in bringing to our attention the problem of variability of clinical electroretinographic testing and the search for standardization.

I should like to acknowledge the collaboration of Harris Ripps, Ph.D., and George Thomas, E.E. in evaluating this paper.

Dr. Burian (Iowa City, Iowa): Dr. Jacobson has had the courtesy of sending me the manuscript of his paper with Mr. Gestring in advance of the

* No cognizance of the baseline is taken in the automatic programmed system which is a severe disadvantage.

meeting. This has given me the welcome opportunity to study the paper and to prepare a few comments, I appreciated this opportunity all the more, since we have been concerned in our laboratory with procedure and equipment design in clinical electroretinography for some years, and more particularly during recent months, because of assignments given us by the new International Society of Clinical Electroretinography.

The problem of standardization is not a new one. It has been considered by a number of workers, but usually from the wrong point of view. The wonderful advances in electronic engineering should not excite us to the extent that we forget that equipment is the servant of procedure. The trend is first to acquire gadgetry and then to develop a procedure which utilizes the equipment.

I believe that our choice of equipment should be determined by the goals of the electroretinogram test. Based on one's knowledge of retinal physiology and electrophysiology one should first ask oneself the question: what is a *reliable, sensitive* and *valid* procedure which will give in the shortest possible time maximum information about the type and degree of abnormality of the electric response of the retina of a patient? Then one should proceed and build one's equipment around the answer obtained. To some extent the essayists have done this. Dr. Jacobson believes that he obtains this maximum information by relying primarily on his "spectro-differential electroretinography." This assumption may be questioned. For example, his program does not allow for sampling the progress of dark adaptation, for repetitive stimulation or for any stress test. Most important, it does not include a period of pre-adaptation to a standardized light. The history of the eyes of different patients during the hours prior to the test differs greatly. The influence of pre-adaptation on the electroretinogram is very considerable, at least during the first 18 to 20 minutes of dark adaptation. We insist, therefore, that a standardized preadaptation treatment of all eye is essential in a standardized procedure.

A few details regarding the instrumentation. I was interested to note that the essayists believe it necessary to interprose fuses to protect the eye. I am not aware of any damage to the subject due to equipment failure reported in electroencephalogram, electrocardiogram, electromyogram, or electroretinogram work, but if it is felt that this danger exists, one would expect that fuses would not prevent it—the eye would be damaged long before the lead has melted. Should protection be really desirable, the use of an electronic power cutoff would be a better solution, or perhaps one could use the new low voltage transistor chopper amplifiers.

The essayists state that oscilloscope recording is essential and that ink writers should be discarded, because of the loss of fine details with the latter. The superiority of the electron beam over the pen needs no emphasis. But I have yet to see an oscilloscope electroretinogram—from our own or any other laboratory—that showed more clinically useful detail than the simultaneously recorded pen written electroretinogram. The necessity of using a camera to record the oscilloscope beam adds a considerable disadvantage to clinical electroretinogram. Unless a Polaroid camera is used, the result is not available for some time and the patient may be irrevocably gone, before the electroretinogram has been read. We have, therefore, adopted in one laboratory the procedure of using only the ink-writer (with a special condensor system giving a relatively high time constant) for routine clinical electroretinogram. For research purposes we combine for a number of reasons simultaneous ink writing and oscilloscope recording, using either a Grass Kymograph camera or a Polaroid camera, in accordance with the requirements of the experiment.

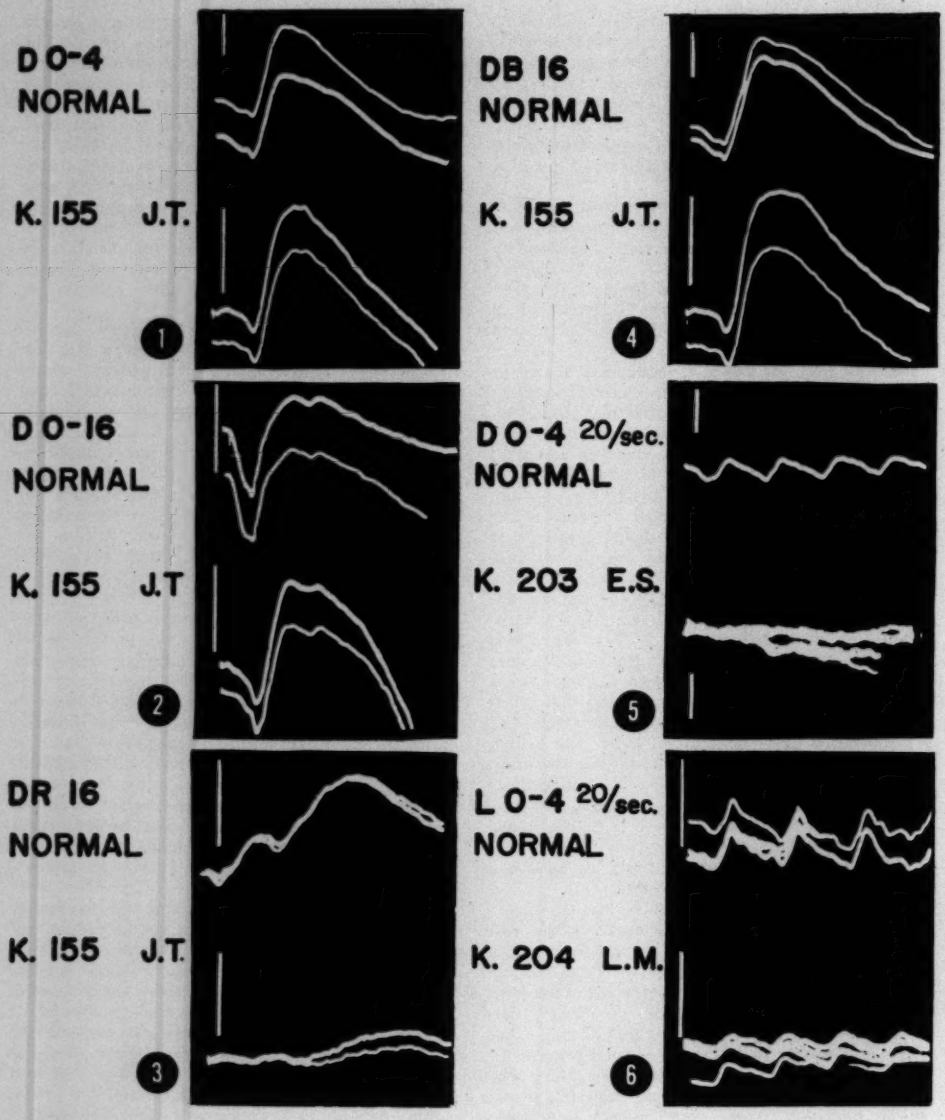
The essayists also object to the use of a stroboscopic light source, meaning probably a gas discharge tube, because of occasional artifacts at the time of firing and because of their spectral composition. Small artifacts do, indeed, occasionally occur, but these are over long before a retinal response is visible. As for the spectral composition, I wonder whether the authors have any evidence regarding their claim that it is not suitable for electroretinogram. It will depend, of course, on the type of tube. François, for example, finds a distinct advantage in using both a Xenon and a Neon filled gas discharge tube and in comparing the results.

Lastly, a word about the electroretinogram-simulator of the authors. The purpose of this device is to check whether a given equipment reproduces a "standard electroretinogram" by properly responding to a photocell, energized in such a fashion as to produce a simulated "standard" electroretinogram. While this is a very ingenious device, a square wave provides a better test of the equipment. Rise time, overshoot, decay, etc. are more easily observed and calculated from the square wave and it is entirely conceivable that a given equipment may faithfully reproduce the simulated "standard" electroretinogram, but not abnormal electroretinograms from patients.

In essence, then, I believe that the choice of equipment should be justified by the procedure, not vice versa. Our present knowledge of retinal potentials does as yet not permit us to fix our electroretinogram procedures for all times. The question of versatility, therefore, looms large in equipment design. I should want to ask the essayists how the diagnostic value of an electroretinogram record is increased by the use of their equipment and how readily it can be modified as our knowledge grows.

DR. ALBERT D. RUEDEMANN, JR. (Detroit): I would like to take just a moment of your time. I certainly think Dr. Jacobson should be complimented for his attempt to bring some rationality to a field that has been flying around in many directions ever since Dewar planted some clay around an eye and poured some saline into it and took an electroretinogram in 1877.

I do think there is something to be said for the technique that Dr. Jacobson has mentioned, and by that I mean the dual being the cathode ray machine



and a definite program in order to reproduce time and again a similar pattern of electroretinographic response.

I must say that we haven't gotten to the fancy electronic device that he has demonstrated here today, but we do use strict adherence to a protocol which has a time factor figured. We have this technique now for a period of over two years, and I feel our results do give us reproducible techniques and reproducible finding over a period of time which can be used to compare cases.

Just by chance I brought some slides along, and I would like to show you some of them. These happen to be selected from a series of individuals who have pigmentary disease or disciform disease of the macula. Several have dissociated angioid streaks.

(Fig. 1) This slide shows how we can demonstrate our method. These are polaroid photographs taken directly from the cathode ray oscilloscope, and this might be one point in the answer to Dr. Burian.

This happens to be the normal, and it shows a response to a moderate intensity stimulus, no filter,

in the dark. The calibration to 200 microvolts is the white line. You see above the normal, taking both eyes simultaneously, the thick line is the left eye and the thin line the right eye. You see above the normal and below the abnormal.

I would like to point out that there is an interesting difference first in the characteristic of the a-wave. You will notice the a-wave makes a rather sharp slope; in this case it seems to rise and then slowly makes a convex curve downward. You will also notice that even though there is a change in the calibration here, there is a rounding of the b-wave.

(Fig. 2) This happens to be the same patient. We have increased the intensity of the stimulus, again in the dark, again with no filter. By the way, this is a standard Grass photic stimulator. Here we notice again the sharp decline in the a-wave, and here we have the convex curve with the slight rounding of the b-wave.

(Fig. 3) In the next slide we have induced in the photic stimulator a standard Grass red filter, and I think the findings here are very obvious. Again this is the same patient whose only clinical finding is a disciform degeneration in the macula, central scotoma, vision reduced to 2/200. No retinal change is observable.

Here is the red response. You can see a very characteristic a-wave, a nice component of the b-wave and a very good b-response. This is the same patient, and you see practically no a-response, a short-slope b, and a much reduced b.

(Fig. 4) This happens to be a standard Grass blue filter inserted, and you see in the normal again the very sharp decline in the a-wave, the rise of the b, and here it is nicely shown in the blue filter. Again the convex character of the a-wave, which is slightly reduced. The a-b ratio is undoubtedly changed, and here you see that this would be called the x-component of the b-wave. Here in the b-filter you see a definite rounding of the b-wave.

(Fig. 5) Here we happen to have a flicker. We do flicker both in the light and in the dark. We do take a response again after flicker to see if there is any change in light sensitivity after flicker.

Fig. 6 is the normal, with a 20 per second flicker, with a very nice, sharp response. In this individual, who happens to be another disciform degeneration with angioid streaks, you see the marked reduction in the flicker response of practically all components, and the flattering of the a-wave here especially. You see the calibration is essentially the same.

I would certainly like to reiterate again that Dr. Jacobson is to be complimented on his attempt to get some order in this particular problem, and I certainly would be willing to go along with that any time.

Dr. K. Nolen Tanner (Portland, Oregon): I, too, feel that a fuse is a relatively poor device to prevent the effects of overload surges. In a measurement which is essentially concerned with potential changes, a simple series resistance, suitably chosen, would serve to limit the current to safe values and would also have the very real function of keeping the input impedance, and time constant, of the amplifier independent of the eye and its connections.

I would also agree that a square wave signal would be the best test of the pass band of the equipment, and it can be readily obtained from commercially available instruments.

Dr. J. H. Jacobson (closing): Dr. Burian let me in on a secret. He told me the way to talk for longer than the time allotted is to get your friends to ask questions. I appreciate that they did.

Let me take these points one by one, if I may.

As far as the questions by Dr. Breinin are concerned, and this also covers some others, the characteristics of the fuse: The fuse we use is a very quick-acting two milliampere fuse. Concerning the necessity for such protection, I can tell you that I had a patient who had both corneas denuded as a result of the need for it.

In trying to determine what caused the damage, we ran a series of animal experiments. I can tell you in answer, in part, to Dr. Burian, definitely and without question that a two milliampere fuse will break long before you will get any damage to the eye. I am not exactly certain that a high resistance will work quite as well, but I think it probably would, except that it would alter the input impedance characteristics of the amplifier.

The question of the ink writer versus the oscilloscope: I will agree that to date we know of no clinical or very few clinical significances that you cannot get with the ink writer. There are exceptions. The fine details shown by Schubert in the electroretinogram of color blindness, and many of the details shown by Dr. Ruedemann, who backed us up very nicely, are absolutely impossible to obtain with an ink writer. The small components of the a-wave cannot be obtained.

I will agree that these are not very many, but I also must say that I feel that unless we do this in the manner that yields the most information we will be ignoring valuable information. I don't think anyone will doubt that the fidelity of an oscilloscope is superior to that of an ink writer. I believe this fidelity is important. I do not believe it wise to ignore details of the curve of the electroretinogram just because we do not yet know their significance.

Concerning the question of flicker, I agree completely that it cannot be obtained with this apparatus. However, I feel that the flicker technique, while extremely important, is most easily obtained with a stroboscope. Our intent with this was to obtain a means of getting a response through a deep red filter. It is impossible to get a response through a No. 70 Wratten filter, which we use with our stroboscope.

The high intensity produced in the longer wave lengths by incandescent bulbs, as compared to the light output characteristics (the spectral composition) of our stroboscope, makes the incandescent bulb better for red stimulus production. We are still performing both techniques, and I feel that this will give us much better information.

Also, by using this technique of filmed recording we are able to superimpose sequential tracing. In this manner we can cancel out our artefact. I am

sure you are all aware that the recording of the electroretinogram is a very important but difficult technique. One of the techniques which has been advocated for it is the use of a frequency analyzer and the use of the stroboscopic flickering light. I don't want to take too much time, but I will tell you that it is our feeling that superimposition of sequential tracing is as good as or better than the use of a frequency analyzer.

The preadaptation is unquestionably important, and I think the program might be altered to do this. We usually keep our patients in the waiting room at a constant level of illumination for a period of half an hour, but I agree this is not ideal.

The question of being able to see the tracing immediately we solve by using a P-7 phosphor on our tube, which allows us to observe it, and for us it has been quite adequate.

The last question by Dr. Burian asked what can be done with this that cannot be done with anything else. We have found it much easier to get a measure of a response using a deep red filter with this technique than with the stroboscope. This we feel is of extreme importance in determining photopic function.

Dr. Ruedemann was the only one to whom I hadn't shown this paper prior to today, and he really helped me enormously, and I want to thank you, sir, for your discussion.

In so far as the simulator is concerned, and its advantages over a square wave generator: To do what can be done with this stimulator one must run a square wave generator at a number of frequencies and test the apparatus at each. This is more difficult to do than to just plug this machine into the input. Also, although since time and other characteristics can be determined with a square wave generator, by using this simulator we are able to judge, more simply and easily, what we are really interested in: the ability of the amplifying and recording system to handle the complex wave that is the electroretinogram.

This device is less expensive to produce than a generator of satisfactory quality, and easier to use in the clinical situation.

THE INFLUENCE OF OXYGEN ON THE PHOTODYNAMIC ACTION OF METHYLENE BLUE ON CATION TRANSPORT IN THE RABBIT LENS*

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We have previously demonstrated that methylene blue markedly reduces cation transport across the lens surface and hence increases lens hydration.[1] This effect is shared by other dyes as well. Among other things these dyes have one feature in common. They demonstrate photodynamic activity. Thus, in the dark this effect of methylene blue is not observed. Light, then, is a necessary feature of this deleterious action of the dyes, at least in low concentrations.

Parenthetically, it might be thought that the photodynamic effect of some agent on the lens would be of particular significance since the lens is continuously exposed to light and the suggestion has been often made that such an exposure may be a factor in the production of cataracts. It is not intended that these experiments be considered to confirm such a theory. Rather, it seems very likely that the photodynamic effect of methylene blue on the lens is similar to that observed on other tissues, on isolated enzymes, proteins, etc. and certainly is not peculiar to the lens itself. The effect, however, does provide an interesting tool for the study of the factors responsible for the maintenance of the lens function.

It should be noted that the poisonous nature of methylene blue may be due to other than photodynamic activity. It seems likely that in higher concentrations other activities of the dye may be manifest and that these do not of necessity require incident light radiation. Thus, in somewhat higher concentrations the dye was found to cause a marked corneal hydration and to block the transport of fluid by which the cornea maintains its normal state of deturgescence.[2] Employing

* From the Department of Ophthalmology, University of Minnesota (Minneapolis) and University of Oregon (Portland) Medical Schools. Supported by grant No. 1979, National Institutes of Neurological Diseases and Blindness, National Institutes of Health, Bethesda 14, Maryland.

these concentrations, no photodynamic effect was observed, that is, the deleterious effect was as marked in the dark as when ambient illumination was allowed.[5]

In the present paper we propose to use those concentrations which have a photodynamic effect. In particular, it is proposed to study the effect of oxygen on the photodynamic activity, since it is generally conceded that in other systems oxygen is required for this activity.[4]

Methods

In general, the techniques employed were those previously described.[5,6] Paired rabbit lenses were placed in separate tubes containing three milliliters of a solution approximately equivalent in ionic composition to aqueous. After refrigeration at 0°C. for 40 to 46 hours one lens was analyzed for sodium, potassium, and water while the other was incubated at 37°C. for an additional six hours before similar analysis. Aseptic precautions were observed. For analysis the lens was dried to constant weight (48 hours) and ashed in the muffle furnace with the aid of a small amount of sulfuric acid. The sodium and potassium analyses were done by flame photometery using a Baird Associates machine.

During refrigeration at 0°C. potassium is lost from and sodium gained by the lens. When subsequently placed at 37°C. potassium reenters the lens and sodium is excreted. The extent to which the lens recovers its normal cation distribution is expressed as the percent recovery* and is considered to be a measure of cation transport against a gradient. It is obvious however, that the steady state obtained represents a balance of two factors, the active transport against a gradient and the movement along a concentration gradient. (The latter factor can be termed "permeability" for the sake of simplicity.) The method employed does not distinguish between the two. Since in most instances the cation exchange during refrigeration is essentially the same, we will consider that any measured change in recovery is due to a decrease in transport.

Methylene blue was added as indicated.* Other additives such as hydrogen peroxide, catalase, and albumin were used as described. These were added after refrigeration and before incubation at 37°C. All solutions were equilibrated with the particular gas mixture under study prior to use. Early in the study nitrogen was used as the diluent gas. As the studies progressed, however, the validity of using commercial preparations of this gas to obtain anaerobic conditions was questioned and helium was substituted.†

Effect of oxygen on cation transport

In previous work we had observed that the cation transport in the lens was inhibited to an appreciable degree under anaerobic conditions.[7,8] This conclusion was also reached somewhat indirectly by Schwartz, Danes, and Leinfelder.[9] In preparation for the current studies, the effect of oxygen was again reevaluated using nitrogen as the diluent (fig. 1). The oxygen tension was observed to have little influence on cation transport. This represented a fairly marked departure from the above mentioned data. Since commercial nitrogen usually contains a small amount of oxygen we switched to helium and found again that cation transport was not influenced by anaerobic conditions (table 1). It is interesting to note that when helium was used, the loss of potassium and the gain in sodium by the lenses during

* Percent recovery is calculated from the expression $\frac{Cr-Ci}{Cr-Cn} \times 100$ where Cr, Ci and Cn are respectively the cation concentrations after refrigeration only, after refrigeration and subsequent incubation at 37°C. and of fresh lenses.

* In calculating the concentration of methylene blue no correction has been made for impurities. The manufacturer's estimate of the methylene blue content of the product employed was 89 percent.

† We wish to express our thanks to Dr. Benjamin Ross, Department of Physiology, University of Oregon Medical School, for preparing and analyzing these gas mixtures for us.

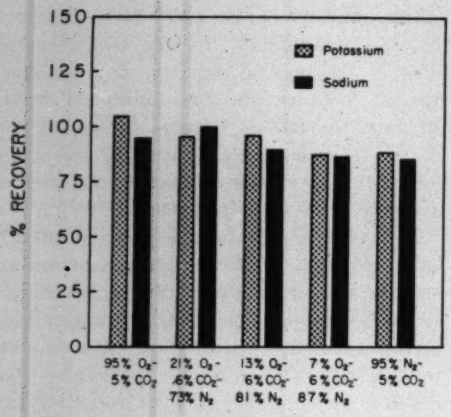

Fig. 1 (Harris, et al.). Effect of various oxygen tensions on cation recovery of rabbit lenses during incubation at 37°C. following a cold-induced cation shift. Each bar represents the average of at least eight experiments.

refrigeration was somewhat less than usually observed when a 95-percent oxygen 5.0-percent CO_2 mixture was used.

We are unable to account for this discrepancy. The previous work had been repeated several times by several individuals as was the present. The major difference in technique lay in the method of preparing the gas mixtures. In our early studies, this was done by displacing water in an appropriate container where the gas volumes could be accurately measured. No analysis of the gas was made prior to its use. In our present series, we have used gas mixtures prepared under pressure in conventional cylinders from a manifold system, analysis being made thereafter. It seems probable that this latter technique provided the atmosphere with the least oxygen contamination. The possibility that a small amount of oxygen might have a deleterious effect has been considered but this seems unlikely. In any event one must conclude that under the conditions of our present experiments, oxygen is not required for cation transport as we measure it.

Effect of oxygen on the action of methylene blue

Methylene blue (5×10^{-5}M) blocks cation transport when the tubes are gassed with 95-percent oxygen and 5.0-percent carbon dioxide. If, however, the oxygen is replaced with helium, cation transport proceeds at its normal pace (table 2). There can be no doubt, therefore, that the effect of methylene blue in this concentration requires oxygen. On the other hand, completely anaerobic conditions were not essential. Thus when 7.0-percent oxygen (the tension of aqueous humor) was used as a gasing mixture a fairly substantial cation transport was observed.

TABLE 1

Effect of various oxygen tensions on cation concentrations and water content of rabbit lenses

Gassing Mixture	Procedure*	No. of Lenses	Potassium meq./1000 gm. Water	Sodium meq./1000 gm. Water	% Recovery K^+	% Recovery Na^+	Water %	Total Base meq./1000 gm. Water	Total Base meq./100 gm. Dry Wt.
	Fresh Lenses	75	123.4± 3.7	22.3± 4.9			66.5±1.3	145.7	28.9
95% O_2– 5% CO_2	1	68	88.8±15.4	59.2±15.4			66.8±1.0	148.0	29.8
	2	69	125.1± 7.7	24.3± 5.2	104.9	94.6	66.0±1.0	149.4	29.0
95% He– 5% CO_2	1	12	101.6± 5.7	45.9± 6.9			66.0±0.2	147.5	28.6
	2	12	128.9± 4.7	23.1± 5.4	125.2	96.6	66.0±0.4	152.0	29.5

* 1—Indicates refrigeration of lenses for 40–46 hours at 0°C.
2—Indicates refrigeration of lenses for 40–46 hours at 0°C. followed by incubation at 37°C. for six hours.

TABLE 2

EFFECT OF VARIOUS OXYGEN TENSIONS ON CATION CONCENTRATIONS AND WATER CONTENT OF RABBIT LENSES INCUBATED IN THE INDICATED CONCENTRATION OF METHYLENE BLUE

Concentration of Methylene Blue	Procedure*	Gassing Mixture	No. of Lenses	Potassium meq./1000 gm. Water	Sodium meq./1000 gm. Water	% Recovery		Water %	Total Base	
						K^+	Na^+		meq./1000 gm. Water	meq./100 gm. Dry Wt.
5×10^{-5} M	1	95% O_2–5% CO_2	19	63.7±26.8	88.5±34.0			67.6±3.0	152.2	31.8
	2		18	50.8±23.5	103.2±26.7	<0.0	<0.0	69.1±3.0	154.0	34.4
5×10^{-4}M	1	7% O_2–6% CO_2	18	78.8± 9.4	67.6± 9.7			66.2±0.8	146.4	28.7
	2	87% N_2	16	117.7± 5.9	27.8± 5.3	87.2	87.9	65.8±0.6	145.5	28.0
5×10^{-5}M	1	95% He–5% CO_2	11	100.9± 7.0	46.3± 8.8			65.6±0.5	147.2	28.1
	2		11	124.2± 4.2	24.0± 4.0	103.6	92.9	65.8±0.4	148.2	28.5
1×10^{-4}M	1	7% O_2–6% CO_2	6	70.2±13.6	76.1±14.7			67.2±1.0	146.3	30.0
	2	87% N_2	6	100.3±11.9	47.7±12.1	56.6	52.8	67.3±1.2	148.0	30.5
1×10^{-4}M	1	95% He–5% CO_2	8	102.9±12.8	46.6± 7.6			65.6±0.7	149.5	28.5
	2		7	123.4± 2.6	22.4± 3.8	100.0	99.6	65.5±0.6	145.8	27.7

*1—Indicates refrigeration of lenses for 40–46 hours at 0°C.
2—Indicates refrigeration of lenses for 40–46 hours at 0°C. followed by incubation at 37°C. for six hours.

If, under anaerobic conditions, the amount of methylene blue was sufficiently increased, cation transport was almost completely blocked (fig. 2). The critical value lay between 1.0×10^{-4}M. and 1.25×10^{-4}M. and was approximately three times the concentration required to block cation transport when 95-percent oxygen was used. It is interesting to note that when a concentration of 1×10^{-4}M. (rather than 5×10^{-5}M.) methylene blue was employed the effect of 7.0 percent oxygen cation transport was more profound (table 2). This would suggest a mass law relationship between oxygen and methylene blue.

INFLUENCE OF HYDROGEN PEROXIDE

Hydrogen peroxide is considered to be formed when certain substances, for example, protein[10] or nicotine[11] are oxidized by methylene blue photodynamically. The lens contains only a small quantity of catalase and thereby may be considered vulnerable to hydrogen peroxide.[12] It seemed advisable to determine whether hydrogen peroxide might be the ultimate toxic substance. Two approaches to the problem were studied, first the effect of added hydrogen peroxide and second, the effect of catalase.

Hydrogen peroxide was added to the media after refrigeration and just prior to incubation at 37°C. to give the final indicated concentrations. In one series (1×10^{-4}M.) the hydrogen peroxide was added in small aliquots each hour during the period of the incubation. It is fairly apparent that in concentrations of hydrogen peroxide which one might anticipate to be formed during radiation of methylene blue, little effect on cation transport was observed. (fig. 3). The substance, therefore, cannot be

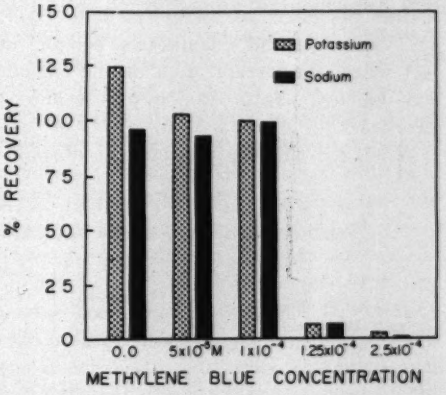

Fig. 2 (Harris, et al.). The effect of various concentrations of methylene blue on cation recovery of lenses in an atmosphere of 95-percent helium, 5.0-percent carbon dioxide during incubation at 37°C. following a cold induced cation shift. Each bar represents the average of at least eight experiments.

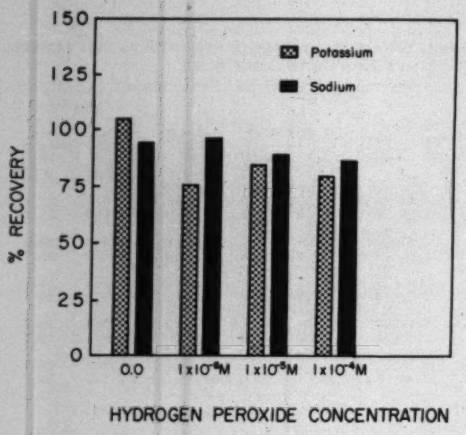

Fig. 3 (Harris, et al.). The effect of various concentrations of hydrogen peroxide on cation recovery of rabbit lenses during incubation at 37°C. following a cold-induced cation shift. Each bar represents the average of at least eight experiments.

considered to be too toxic to the system when supplied in the external media.

In another series, catalase was added to the incubating medium after refrigeration and just prior to subsequent incubation at 37°C. The enzyme did not alter normal cation transport (table 3). However, it was observed to afford a partial protection against the effect of methylene blue (table 4). There was no quantitative benefit. In fact when the concentration of the enzyme was doubled, less protection was seemingly afforded.

When the catalase was boiled, substantially the same effect was observed. This led us to the conclusion that the protein nature of catalase rather than its enzymatic activity was the determining factor. To test this point a molar equivalent amount of albumin was added after refrigeration and before incubation. It was found to have approximately the same protective effect as the catalase itself. It seems unlikely therefore, that the protective action of the latter is due to its enzymatic activity.

It is of interest to note that this protective effect of protein was also noted against the enzymolytic action of methylene blue. As Fowlkes[13] has demonstrated lenses immersed in methylene blue and exposed to light lose dehydrogenase activity identified by Nitro-BT (a tetrazolium derivative). Addition of albumin in the amount indicated here protects against this loss.

Comment

It seems reasonable from this series of studies that under the conditions of our experiments, oxygen is not required for cation transport and the maintenance of normal hydration. We are unable to explain the difference between results here presented and those which we have published in the past. Artefactual variance would most likely have occurred in the previous rather than the present studies. The thought that oxygen is not a necessary metabolite of the lens is not a new one. There is evidence which in-

TABLE 3

Effect of catalase on cation concentrations and water content of rabbit lenses

Catalase Concentration	Procedure*	No. of Lenses	Potassium meq./1000 gm. Water	Sodium meq./1000 gm. Water	% Recovery K^+	% Recovery Na^+	Water %	Total Base meq./1000gm. Water	Total Base meq./100 gm. Dry Wt.
0.0	1	68	88.8±15.4	59.2±15.4			66.8±1.0	148.0	29.8
	2	69	125.1± 7.7	24.3± 5.2	104.9	94.6	66.0±1.0	149.4	29.0
10 Units— 0.1 mg./3 ml.	1	8	86.4± 6.5	53.1±10.0			66.5±0.9	139.5	27.7
	2	8	121.0±11.5	22.9± 9.8	93.5	98.1	66.7±1.7	143.9	28.8

* 1—Indicates refrigeration of lenses for 40–46 hours at 0°C.
2—Indicates refrigeration of lenses for 40–46 hours at 0°C. followed by incubation at 37°C. for six hours.

TABLE 4

EFFECT OF CATALASE AND PROTEIN ON THE CATION CONCENTRATIONS AND WATER CONTENT OF RABBIT LENSES INCUBATED IN 5×10^{-5} M. METHYLENE BLUE

Additive	Procedure*	No. of Lenses	Potassium meq./1000 gm. Water	Sodium meq./1000 gm. Water	% Recovery		Water %	Total Base	
					K^+	Na^+		meq./1000 gm. Water	meq./100 gm. Dry Wt.
None	1	19	63.7±26.8	88.5±34.0			67.6±3.0	152.2	31.8
	2	18	50.8±23.5	103.2±26.7	<0.0	<0.0	69.1±3.0	154.0	34.4
Catalase 10 Units—0.1 mg./3 ml.	1	11	78.4±18.7	63.0±22.8			66.5±1.9	141.4	28.1
	2	11	110.0±18.3	34.8±19.3	70.2	69.3	66.7±1.6	144.8	29.0
Catalase 20 Units—0.2 mg./3 ml.	1	9	46.5±14.0	105.8±18.1			69.3±1.7	152.3	34.4
	2	10	65.8±31.5	84.0±35.4	25.1	26.1	69.6±2.4	149.8	34.3
Boiled Catalase 10 Units—0.1 mg./3 ml.	1	9	77.7±18.3	61.6±24.0			67.6±2.4	139.3	29.1
	2	10	94.8±29.4	41.8±23.7	37.4	50.4	67.2±2.3	136.6	28.0
Albumin 0.065 mg./3 ml.	1	10	57.7±14.7	92.0±17.8			67.9±1.2	149.7	31.7
	2	10	71.4±31.0	72.3±34.4	20.9	28.3	69.1±2.2	143.7	32.1

* 1—Indicates refrigeration of lenses for 40–46 hours at 0°C.
2—Indicates refrigeration of lenses for 40–46 hours at 0°C. followed by incubation at 37°C. for six hours

dicates that the majority of the energy produced by the lens is provided by anaerobic metabolism.[14]

The oxygen dependence of methylene blue in the concentrations utilized fits the data which has been observed using other tissues. It should be emphasized that this oxygen dependence bears no relationship to the question of the oxygen need of the lens under normal circumstances. The measured response of the lens to the photodynamic activity of methylene blue most likely results from oxidation of some key enzyme or protein barrier. Any hydrogen peroxide which may be formed during the oxidation is probably of no biological significance.

The reasons for the protective effect of proteins, although fairly small, is not too clear. It may relate to the fact that they provide an alternate substrate. Likewise, a methylene blue-protein complex may be formed and the availability of methylene blue thereby reduced.

The findings here described further emphasize the fact that methylene blue has multiple actions. These have been discussed previously and need not be reiterated in detail.

They include the effect of the dye as an uncoupling agent as well as its possible influence on a redox pump in the manner suggested by Conway and Kernan.[15] We know of no published evidence that either of these effects requires light but have observed a photodynamically induced reduction in the high energy phosphate content of lenses incubated at 37°C. for 24 hours in methylene blue solution.[16] Whether this is a true uncoupling reaction cannot be stated at the moment. Nonetheless, the decreased availability of high energy phosphates is undoubtedly of significance since the immediate energy required for transport probably comes from these linkages. Although such findings tend to emphasize the effect of this agent on transport, the possibility mentioned previously[1] that oxidation of a protein constituent at a limiting barrier may permit a more rapid diffusion along a concentration gradient cannot be ignored.

Summary

1. In contrast to previous findings, oxygen has not been found to be essential for cation transport across lens surfaces in an acute experiment.

2. The photodynamic action of methylene blue on cation transport is oxygen dependent.

3. High concentrations of methylene blue block cation transport in the absence of oxygen.

4. Any hydrogen peroxide produced during photodynamic oxidation does not alter cation transport.

5. Small amounts of protein in the external media partially protects the lens against the photodynamic action of methylene blue.

6. The photodynamic effect of methylene blue results from the oxidation of a key enzyme (or enzymes) and protein constituents of a limiting barrier.

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Discussion

Dr. Carl Wachtl (Detroit): The term "photodynamic action" or "photodynamic phenomenon" is a translation of a German expression coined by Tappeiner. It has been applied to cases wherein a substance, usually a dye or a pigment, sensitizes a biological system to light and causes a photochemical reaction in which molecular oxygen takes part.

Dr. Harris had shown previously that a cation shift occurs in the lens upon refrigeration which is reversed if the refrigerated organ is incubated at 37°C. under physiologic conditions. Methylene blue in a concentration of five times 10^{-5} molar prevents the reversal of cation shift after refrigeration provided the system is illuminated and 95-percent oxygen is present.

Thus, the authors have demonstrated that the criteria for photodynamic action of the dye on cation transport in the lens are fulfilled. When the gas

mixture contained only seven-percent oxygen, a concentration believed to lead to an oxygen tension as exists in the aqueous humor, the inhibition was very slight; there was also no inhibition.

Another effect of methylene blue on the lens was demonstrated by Dr. Constant, who cultured rabbit lenses in the presence of air. She observed that five times 10^{-5} molar methylene blue inhibits mitosis and causes the formation of vacuoles. This fits in well with the remarks of Dr. Dische, who suspects that the SH groups may be involved. According to the theory of Mazia on mitosis, the SH groups are a vital part of the mitotic mechanism.

It would be interesting to learn if this inhibition of mitosis also occurs in the dark. I wonder if Dr. Harris has noticed an effect of the dye on the cation balance of lenses incubated at 37°C. without prior refrigeration. Formation of the complex between the dye and the protein, as mentioned by Dr. Fowlks, would make a protective action of the latter understandable.

In the course of this research it was found that a reversal of the cation shift after refrigeration occurs also in the absence of oxygen. The authors had come to the opposite conclusion on the basis of previous results. This is certainly somewhat surprising. I could think of two possible explanations, namely, that the cation transport against the concentration gradient, as occurs in the case of sodium, either does not require optimal conditions of metabolic energy production, or that the lens has stored enough energy to restore the cation balance over a period of six hours.

An experiment wherein lenses are incubated after refrigeration at 37°C. for longer periods may facilitate a distinction between these two possibilities.

The authors have, with the use of their proven technique, made another advance toward an understanding of the factors responsible for lens function.

Dr. John E. Harris (closing): I wish to thank the discussers for their excellent ideas.

To answer some specific questions; first, Dr. Wachtl asks whether it is necessary to refrigerate these lenses to demonstrate the photodynamic effect of methylene blue? No, it isn't. One can demonstrate it by simply incubating at 37°C. for some period of time. When light is excluded normal cation balance is maintained. Where light is permitted, there is a loss of potassium and gain of sodium by the lens.

As a matter of fact, the variety of factors which, over a period of years, we have shown to influence lenticular cation content and hydration can be measured by other techniques. We use the temperature-reversible technique simply because it seems to provide a more sensitive measure of these changes.

As to Dr. Constant's studies, I believe last year she did state that when the lenses were protected from light vacuoles normally observed when lenses were incubated with methylene blue did not appear. I do not know, however, whether she found a photodynamic effect on mitotic activity.

I am equally puzzled by our data on the influence of oxygen. I think the answer lies somewhere in our experimental situation. We have been much more exacting in the preparation of our gas mixtures in recent years. Even so, careful scrutiny of the original protocols in which we found the cation transport to be oxygen sensitive has failed to reveal any source of experimental error.

Dr. Wachtl suggested that possibly there is sufficient, available energy left after 40 hours refrigeration to provide all that is needed during the succeeding six hours at 37°C. I don't believe this is true. We have tried such a variety of other enzyme and metabolic poisons and have found that in their presence one can completely block cation transport. All I can say is that in certain acute situations the lens can survive without oxygen according to our methods of measuring survival.

The last question by Dr. Balazs was very interesting. He points out that if an oxidation-reduction reaction is responsible for the effects here observed, the reacting compounds should have potentials fairly close to one another. Actually, as you know, a wide variety of dyes show photosensitizing effects, and these dyes vary markedly in their particular position in the oxidation-reduction potential spectrum. This again would suggest that we must be dealing with a variety of reactions.

INFLUENCE OF RETINAL ADAPTATION UPON THE PUPILLARY REFLEX TO LIGHT IN NORMAL MAN*

PART I. EFFECT OF ADAPTATION TO BRIGHT LIGHT ON THE PUPILLARY THRESHOLD

OTTO LOWENSTEIN, M.D., AND IRENE E. LOEWENFELD, PH.D.
New York

In the first part of a study of the relations between pupillary responses and visual sensation[1] we examined the pupillary reactions of the dark-adapted eye to intermittent light stimuli of threshold intensity.

The pupillary threshold for white light was found to be about one log unit higher than the subjects' absolute visual threshold. Correspondingly, the pupillary threshold for red light was slightly higher than the visual threshold for red light. When intermittent white light stimuli of increasing intensity were presented, it was found that pupillary reactions evoked by light stimuli below the photopic threshold showed a long latency period, low contraction speed, and small extent. When the light stimulus was prolonged beyond 1/10 second, the pupillary contraction was not prolonged. In contrast, pupillarly reactions to white light stimuli above the photopic threshold showed a shorter latency period, a faster, more extensive contraction, and a great increase in extent and duration of the contraction when the light stimulus was prolonged.

We concluded that both the retinal rods and cones furnish afferent impulses for the pupillary reflex to light. The cone-function and rod-function have separate thresholds, just as for vision. The cones, though less sensitive, are more effective than the rods in producing an extensive and prolonged pupillary response.

The present paper is concerned with a more detailed comparison of the pupillary reactions elicited by white and by red light stimuli of low intensity, and with a study of the effect of adaptation to bright light upon these responses.

METHOD

As in the previous experiment,[1] the pupillogram was recorded while series of intermittent light stimuli were presented. These records were obtained with the aid of the electronic pupillograph,[2] an infrared-sensitive scanning device which furnishes accurate, continuous traces of the pupillary diameter and reactions to light or other stimuli in visual darkness or at any level of light adaptation.

The subjects' eyes were adapted to darkness, to bright white light or to bright red light (cf. below). The light source for the adapting light and for the test stimuli was a Sylvania glow modulator tube which was used in the same manner as before.[1] For the purpose of the present study, test stimuli of one second or of 1/10 second duration were used in four-second cycles, that is, with three-second dark-intervals between one-second light stimuli and with 3.9-second dark intervals between 1/10-second light stimuli. Light intensity was controlled by Wratten gray filters, graded in 0.1 log unit steps. For red light, the stimulating light was passed through a Wratten No. 92 red filter.

Five normal subjects, aged 17 to 34 years, were used. Four of them, namely the subjects A, B, C and D, had been subjects in the previous experiments. On each experimental day, the subject's visual threshold was determined within 1/10 log unit, for white and for red light stimuli of 1/10 second and of

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1 second duration. The intensity of light stimuli used for each subject was based on the visual and pupillary thresholds of the subject rather than on a fixed brightness value.*

Results

A. Normal pupillary reflexes of the dark-adapted eye, elicited by white and by red light stimuli of low intensity

1. White-light and red-light visual and pupillary thresholds

To test the visual threshold, we presented 10 consecutive white or red test stimuli of one second or of 1/10th second duration in four-second cycles and noted the number of flashes which were seen in each series. Three such test-series were presented each time.

The visual thresholds of our subjects for white light stimuli of 1 second duration ranged between $10^{-9.6}$ and about $10^{-9.5}$, that is, none of the flashes were seen with Wratten grey filters of $10^{-9.6}$ transmission while from two to 10 of the flashes were seen with Wratten filters of $10^{-9.5}$ transmission. The threshold for red light stimuli of one-second duration varied between $10^{-6.1}$ and $10^{-6.2}$.

While the visual responses to one-second light stimuli were fairly uniform from experiment to experiment and from subject to subject, the threshold for 1/10th second stimuli was more variable. In subjects B, C and D, the threshold for the shorter white and red stimuli was only a little higher than for the longer stimuli. The difference was slightly greater in subject E and much greater in subject A (tables 1A and 1B).

The pupils of all our subjects failed to respond to light stimuli of visual threshold intensity. Subjects B and C reacted well to white test stimuli of 10^{-9} intensity, that is, 0.5 log unit brighter than the absolute visual threshold. In subject E the light intensity required for pupillary contractions was $10^{-8.5}$ and in subject A and D 10^{-8}. A mechanism partly responsible for these individual differences will be described later.

The difference between the intensity needed for visual and for pupillary responses was smaller for red than for white test stimuli. All subjects showed distinct pupillary contractions when the stimulus intensity was raised 0.5 log unit above the visual threshold for red light.

2. The latency period of low-intensity reactions to light

As described before,[1] pupillary reactions elicited by light stimuli of low intensity are characterized by a long latency period, low speed, small extent and short duration. As an example, Figure 1 shows pupillary reactions of subject C in response to light stimuli of 1/10th second duration for five brightness levels.

The latency period of the reactions decreased progressively from 0.55 second in the threshold reaction *a* (stimulus intensity 10^{-9}) to a minimal value of 0.27 second in *d* and *e* (20 percent and 100 percent stimulus intensity respectively). Since the extent of the contractions increased progressively from 0.25 mm. in *a* to 2.25 mm. in *e*, the question arose whether the latency period of a given reflex depended primarily upon the extent of the reaction or upon the intensity of the stimulating light.

* As explained in the earlier paper, we used the glow modulator tube as a flashing light at 100 cycles per second, with five millisecond flash duration. The time-characteristics of the light flashes were measured with a vacuum-photocell and oscilloscope while the stability of the light was checked with a selenium barrier layer cell and milliamperemeter. Until a measurement of the absolute light intensity can be obtained, we will continue to express light intensity in terms of (1) an arbitrary scale of gray filters used in our experiments, with 100% of the full intensity of the light source and (2) logarithmic units above each subject's visual threshold. For example, the visual thresholds found in the previous experiment (10^{-10}) were only seemingly lower than the ones described in the present paper ($10^{-9.5}$-$10^{-9.6}$). The glow modulator tube used during the second experiment proved somewhat less efficient than the one used in the previous work; thus, less gray filters were needed to reach the visual threshold.

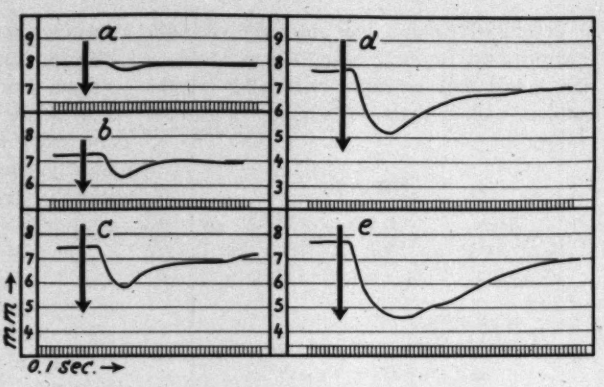

Fig. 1. (Lowenstein and Loewenfeld.) LATENCY PERIOD AND DURATION OF PUPILLARY REACTIONS TO LIGHT STIMULI OF VARIOUS INTENSITIES.

Pupillogram of a normal, 17-year-old girl (subject C). Pupillary diameter is plotted as the ordinate (in mm.) against time as the absissa (in 0.1 second units). The record of the left pupil only is shown.

In all experiments, the eye was fully dark-adapted. The full intensity of the 1/10 second light stimuli was used in experiment *e*. In the other experiments, the light intensity was reduced by Wratten gray filters, whereby the transmission was 19^{-6} in *a*, 10^{-7} in *b*, 10^{-8} in *c* and 20% in *d*.

The latency period of the reactions shortened progressively with increasing stimulus intensity; in contrast, the duration of the contractions remained the same until bright light was used.

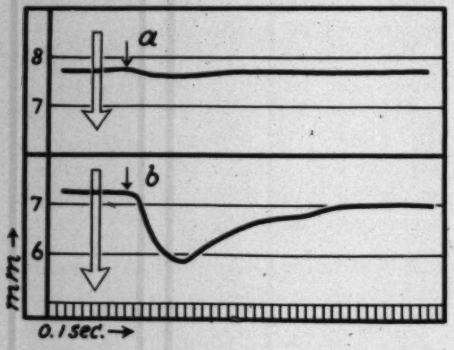

Fig. 2 (Lowenstein and Loewenfeld). EXTENT OF CONTRACTION VERSUS LATENCY PERIOD OF LOW-INTENSITY LIGHT REFLEXES.

Pupillogram of low-intensity light reflexes (10^{-6}) in two normal subjects. Pupillary diameter is plotted as the ordinate (in mm.) against time as the abscissa (in 0.1 second units). The pupillogram of the left eye is shown in each case. The white arrows indicate the duration (1/10 second) of low intensity light stimuli. The small black arrows show the end of the latency period of each reflex.

(*a*) The subject was a tense, excitable young woman of 22, with strong supranuclear inhibition of the third nerve nucleus.

(*b*) Pupillogram of a 17-year-old girl with less inhibition of the light reflex.

The latency period was alike in the two subjects, even though the extent of reaction *a* was only 0.15 mm., while the reaction *b* was almost 10 times as extensive (1.4 mm.). (For further discussion, see text.)

This question was answered by the fact that reactions with different extent often have the same latency period. When series of light stimuli of a given intensity are presented, the extent of the pupillary contractions varies considerably from reaction to reaction and from individual to individual. As pointed out before,[1, 3] such variations in the extensiveness of the parasympathetic light reflex may be caused by differences in the degree of supranuclear inhibition. Cortico-thalamic-hypothalamic mechanisms are brought into play by psycho-sensory stimuli, by spontaneous thoughts and by emotions. These mechanisms affect the pupil, firstly, by inhibiting the oculomotor nucleus and, secondly, by sending impulses via the cervical cord and peripheral sympathetic chain to the dilator muscle of the pupil. Both these influences tend to diminish the extent of pupillary contractions. Tense, emotionally excited subjects therefore show less extensive reactions to weak light stimuli than relaxed or tired subjects. In contrast, the latency period of the reflexes is not greatly affected by the emotional state except in cases of extreme supra-nuclear irritation.

For example, Figure 2,*a* shows the reaction to a low-intensity light stimulus in subject D, a tense, excitable young woman of 22. Figure 2,*b* shows a reaction to a similar light stimulus in subject C, a 17-year-old girl with average supra-nuclear inhibition. The lat-

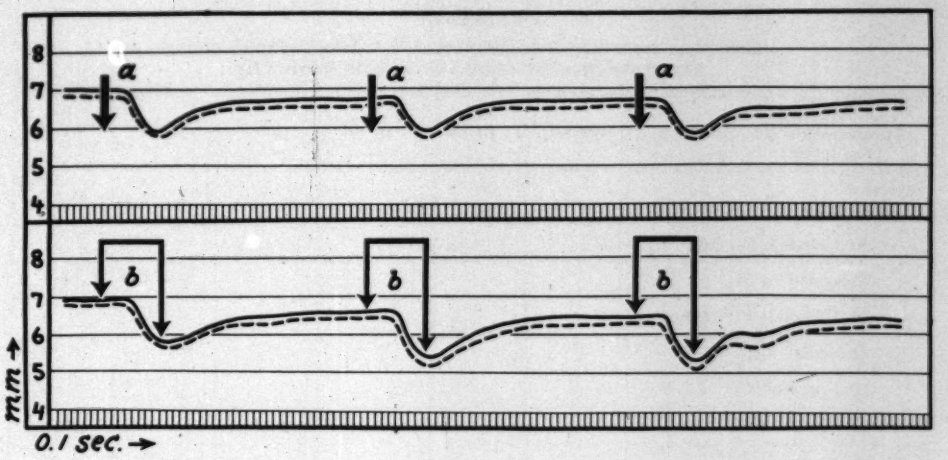

Fig. 3 (Lowenstein and Loewenfeld). PUPILLARY REACTIONS TO LOW INTENSITY LIGHT STIMULI OF ONE TENTH AND OF ONE SECOND DURATION.

Pupillogram of a normal 24-year-old woman. Pupillary diameter is plotted as the ordinate (in mm) against time as the abscissa (in 0.1 log unit steps). The solid line represents the right, the broken line the left pupil. The small arrows *a* show the moments when light stimuli of 1/10 second duration and of low intensity (10^{-1}) were presented. The double-arrows *b* show time of elicitation of one-second light stimuli of the same intensity. The longer light stimuli did not prolong the pupillary reactions.

ency period of the two reactions was alike, even though the reflex *b* was almost 10 times as extensive as the reflex *a*.

In all our subjects, reactions to threshold-intensity white light stimuli showed latency periods similar to those found in reactions to threshold-intensity red light stimuli (for example, Figure 6, *a* and *b*).

3. *The duration of low-intensity reactions to light*

When the dark-adapted eye is exposed to white light stimuli below the photopic threshold, the pupillary contractions are not prolonged by extending the light stimulus from 1/10th of a second to one second (fig. 3). In contrast, light stimuli above photopic threshold brightness and of one second duration cause much longer, more extensive contractions than stimuli of equal intensity but shorter duration.

Since a sharp increase in the duration of the responses to one-second white light stimuli was found at a brightness level just above the photopic threshold, it was thought possible that the prolonging-effect might be a specific property of the pupillary cone-function rather than a quantitative effect in the neural reflex arc. However, pupillary reactions to threshold intensity red light stimuli, just as the white-light threshold reflexes, failed to show the prolonging effect. At present we are therefore unable to explain this interesting phenomenon.

4. *The variability of low-intensity reactions to light, and the fading effect*

It has already been stated that low-intensity reactions to light vary from subject to subject and from reaction to reaction, according to (1) the subject's visual threshold and (2) fluctuations in the degree of supranuclear inhibition. When series of light stimuli of various intensities are presented, individual reactions to light stimuli of very low intensity may be as extensive, or they may even exceed the extent of individual reactions elicited by stimuli of higher intensity. When, however, our subjects were examined repeatedly, it appeared that the general pat-

TABLE 1A

VISUAL THRESHOLD FOR WHITE LIGHT STIMULI OF ONE-TENTH AND OF ONE SECOND DURATION (TEN EXAMINATIONS IN SUBJECT A)

Light Intensity (transmission of gray filters)		10^{-9}	$10^{-9.1}$	$10^{-9.2}$	$10^{-9.3}$	$10^{-9.4}$	$10^{-9.5}$	$10^{-9.6}$
Subject A								
1	1 second						8+ 2−*	10−*
	0.1 second	10+	10−					
2	1 second						7+ 3−	10−
	0.1 second	6+ 4−	10−					
3	1 second						6+ 4−	2+ 8−
	0.1 second	2+ 8−	10−					
4	1 second						5+ 5−	10−
	0.1 second	10+	3+ 7−					
5	1 second						2+ 8−	10−
	0.1 second	10+	4+ 6−					
6	1 second						2+ 8−	10−
	0.1 second	4+ 6−	10−					
7	1 second						2+ 8−	10−
	0.1 second	3+ 7−	10−					
8	1 second						4+ 6−	10−
	0.1 second	6+ 4−	10−					
9	1 second						8+ 2−	10−
	0.1 second	10+	4+ 6−					
10	1 second						7+ 3−	10−
	0.1 second	5+ 5−	10−					

tern of responses shown by each subject was repeated with reasonable reliability. Figure 4 shows the variability of pupillary contractions of subject E, elicited by series of white and of red light stimuli of three brightness levels. The series *a* and *b* were recorded on the same day, the series *c* and *d* a week later. We have repeated such experiments with similar results up to 18 times over a period of three months.

Figure 5 shows the variations in similar series of light reflexes of subjects A and B. It will be noted that in subject A the first of each series of ten reactions was always the most extensive one and that the responses which followed declined rapidly. In contrast, the reactions of subject B showed no such fading upon repeated elicitation, and the first reaction of a series was usually not the most extensive one.

Subjects C, D and E showed the fading effect to a lesser degree. It was absent in many series of reactions (fig. 4), but when averages were computed of ten or more series of

TABLE IB

VISUAL THRESHOLD FOR WHITE LIGHT STIMULI OF ONE-TENTH AND OF ONE SECOND DURATION (TEN EXAMINATIONS IN SUBJECT B)

Light Intensity (transmission of gray filters)		10^{-9}	$10^{-9.1}$	$10^{-9.2}$	$10^{-9.3}$	$10^{-9.4}$	$10^{-9.5}$	$10^{-9.6}$
Subject B								
1	1 second						10+	10−
	0.1 second						9+ 1−	10−
2	1 second						10+	10−
	0.1 second						8+ 2−	10−
3	1 second						10+	10−
	0.1 second						10+	10−
4	1 second						10+	10−
	0.1 second						7+ 3−	10−
5	1 second						10+	10−
	0.1 second						10+	10−
6	1 second						10+	10−
	0.1 second						7+ 3−	10−
7	1 second						10+	10−
	0.1 second						8+ 2−	10−
8	1 second						10+	10−
	0.1 second						9+ 1−	10−
9	1 second						9+ 1−	10−
	0.1 second						3+ 7−	10−
10	1 second						10+	10−
	0.1 second						10+	10−

*= + indicates light flashes seen, − light flashes not seen within a series of 10 consecutive test flashes.

experiments, the averages of the first reactions of all series were slightly greater than those for later responses. In each subject, the fading effect was equally pronounced for reactions to white and to red low-intensity light.

We do not know the mechanism of the fading effect. It is likely to be a retinal rather than a midbrain phenomenon, since subject A's visual responses faded in a similar way as her pupillary reactions. We tend to regard this symptom as undesirable, because subject A, who showed it most distinctly, had, among our subjects, the greatest disparity of the visual thresholds for long and for short light stimuli (table 1A), a relatively high pupillary threshold *without* unusually great supranuclear inhibition, and the greatest variability of both visual and pupillary responses. In contrast, subject B, (table 1B) who showed the fading effect least, had low visual thresholds for short

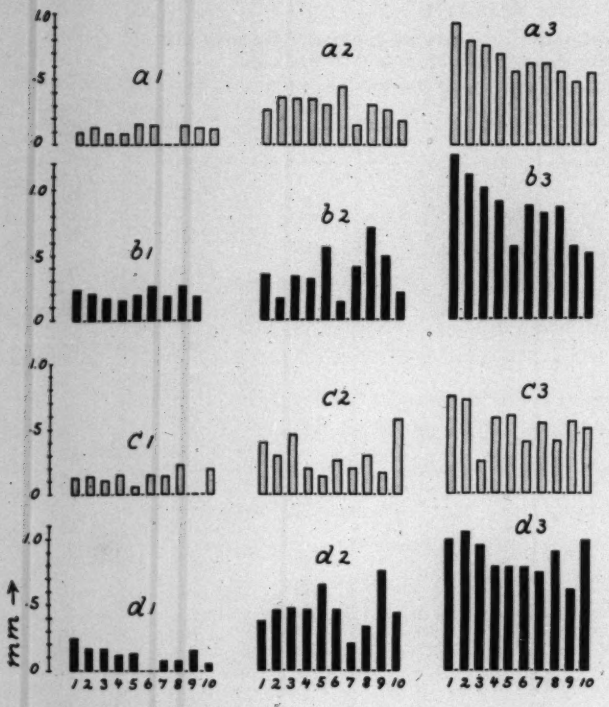

Fig. 4 (Lowenstein and Loewenfeld). EXTENT OF PUPILLARY CONTRACTIONS ELICITED BY SERIES OF RED AND OF WHITE LIGHT STIMULI OF LOW INTENSITY.

Data derived from the pupillogram of a normal 34-year old man, after complete dark adaptation. The height of the columns indicates the extent of individual pupillary contractions (in mm.). The reactions were elicited by series of 10 consecutive light stimuli of one second duration, with three-second dark intervals between stimuli. The grey columns represent reactions to white light stimuli, the black columns reactions to red light stimuli. The following stimulus intensities were used:

a 1 and c 1. White light stimuli at pupillary threshold ($10^{-8.5}$), one log unit brighter than the subject's visual threshold for white stimuli ($10^{-9.5}$).

b 1 and d 1. Red light stimuli at pupillary threshold ($10^{-5.1}$), one half log unit brighter than the subject's visual threshold for red stimuli ($10^{-6.1}$).

a 2 and c 2, a 3 and c 3. One and two log units respectively brighter than a 1 and c 1 ($10^{-7.5}$ and $10^{-6.5}$).

b 2 and d 2, b 3 and d 3. One and two log units respectively brighter than b 1 and d ($10^{-4.1}$ and $10^{-3.1}$).

The extent of the reactions fluctuated from reaction to reaction within each series. On the average, the pupillary reactions were, however, consistent for each brightness level (see text).

light stimuli, low pupillary thresholds and, among the five subjects, the most reliable visual and pupillary responses.

5. The relative effectiveness of red and of white low-intensity light stimuli

When the intensity of white or of red light stimuli was increased above the value needed for pupillary threshold responses, the pupillary contractions became faster and more extensive. This increase in function was more marked when red than when white light stimuli were used (figs. 4, 5, and 6).

The cause of the greater increment of pupillary activity for a given increment of red stimulus intensity, compared with the effectiveness of the same increment of white light intensity, is unknown.

B. EFFECT OF ADAPTATION TO BRIGHT LIGHT UPON THE PUPILLARY LIGHT REFLEX

If we wish to use the pupil as an indicator of changes in retinal sensitivity brought about by adaptation to light, two problems are encountered:

1. Figure 7 shows how the extent of the pupillary light reflex decreases when the stimulus intensity is reduced in 1-log unit steps, from full intensity to the absolute visual threshold. When we consider the vast range of light intensities used in these experiments (1000,000,000 : 1), the decrease in the extensiveness of the pupillary responses is not very great (18 : 1). The diameter of the pupil after adaptation to light of various brightness levels changes even

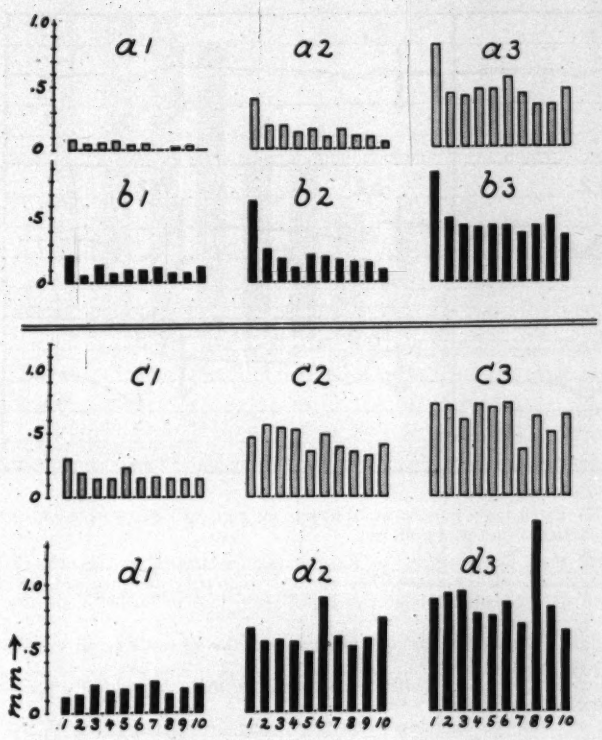

Fig. 5 (Lowenstein and Loewenfeld). DECLINE OF PUPILLARY REACTIONS TO REPEATED LIGHT STIMULI OF LOW INTENSITY.

The height of the columns indicates the extent (in mm.) of the pupillary contractions elicited by series of 10 consecutive 1/10 second light stimuli, with 3.9-second dark intervals between stimuli. The grey columns represent white-light responses, the black columns red-light responses. The eyes had been dark-adapted.

Series *a* and *b* were obtained from subject A, a 25-year-old woman with relatively high pupillary threshold (10^{-4}), irregular responses and relatively high visual threshold for short light stimuli (table 1). Series *c* and *d* were obtained from subject B, a 24-year-old woman with low pupillary threshold (10^{-5}), reliable responses and low visual threshold for short light stimuli. The stimulus intensities were:

a 1 and c 1. Pupillary threshold for white light (10^{-4} in *a* and 10^{-5} in *c*).

a 2 and a 3, c 2 and c 3. One and two log units respectively brighter than *a 1* and *c 1.*

b 1 and d 1. Pupillary threshold for red light (10^{-3} in *b*, $10^{-3.7}$ in *d*).

b 2 and b 3, d 2 and d 3. One or two log units respectively brighter than *b 1* and *d 1.*

The reactions of subject A declined rapidly, and the first response in each series was always the largest one. In contrast, the reactions of subject B showed no such decrease (see text).

less than the extent of one-second light reflexes, because the pupil redilates partly during light adaptation. For these reasons, differences in pupillary diameter, or in the extent of the light reflex cannot be considered as delicate indicators for retinal events.

On the other hand, we had found the pupillary threshold to be sensitive to differences in light intensity of fractions of a log unit. We therefore decided to use the changes in the pupillary threshold brought about by various conditions of light adaptation as indicators of retinal sensitivity.

2. The pupillary behavior of the dark-adapted eye differs completely from the pupillary behavior of the light-adapted eye. The pupils are large and quiet in darkness. When one or both eyes are exposed to a constant light stimulus, the pupils contract, then redilate partially and begin to oscillate (fig. 8). In the literature, such oscillations were often named "pupillary unrest" or "hippus." Several theories were proposed to explain their mechanism, and their presence or their absence were thought to indicate various pathological conditions. A discussion of these theories would exceed the scope of the present paper.

We have recorded such oscillations in all normal subjects, for periods up to 90 minutes. As far as we know, they continue indefinitely. The movements are irregular and measure up to about one-half to two mm. in extent, with the fastest rate of oscillation

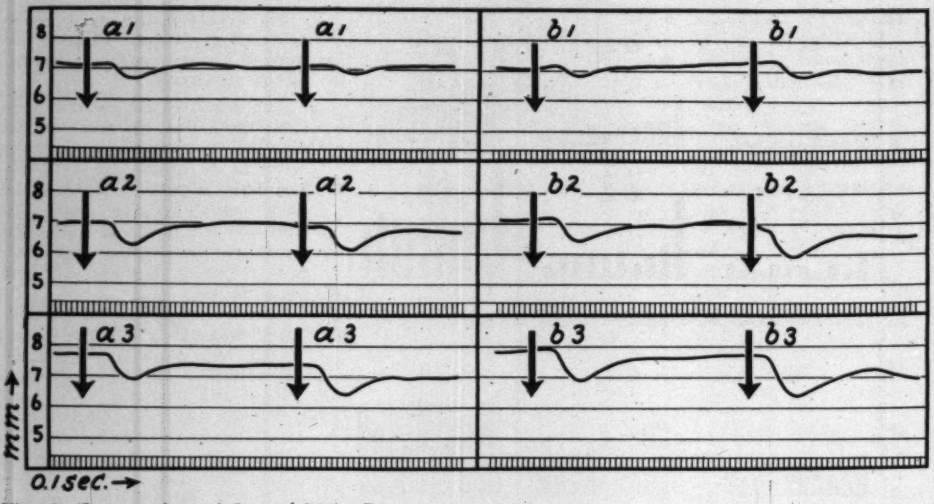

Fig. 6 (Lowenstein and Loewenfeld). PUPILLARY REACTIONS ELICITED BY RED AND BY WHITE LIGHT STIMULI OF LOW INTENSITY.

Pupillogram of a 34-year-old normal man. The diameter of the left pupil is plotted as the ordinate (in mm.) against time as the abscissa (in 0.1 second units). The eye was completely dark-adapted. The arrows indicate the time of elicitation of 1/10 second light stimuli, whereby the following intensities were used:

a 1. Pupillary threshold for white light ($10^{-8.5}$), one log unit brighter than the subject's visual threshold for white light stimuli of 1/10 second duration ($10^{-9.5}$).

b 1. Pupillary threshold for red light ($10^{-5.7}$), one half log unit brighter than the subject's visual threshold for red light stimuli of 1/10 second duration ($10^{-6.2}$).

a 2 and a 3. White light stimuli, one and two log units respectively brighter than *a 1* ($10^{-7.5}$ and $10^{-6.5}$).

b 2 and b 3. Red light stimuli, one and two log units respectively brighter than *b 1* ($10^{-4.7}$ and $10^{-3.7}$).

The white-light and red-light threshold reactions showed the same speed, extent and latency period. With increasing intensity, the red light stimuli proved somewhat more effective than the white stimuli, so that the red-light reactions in *b 2* and *b 3* were more extensive and faster than the white-light reactions in *a 2* and *a 3*.

about two per second when bright light is used, while extent and frequency diminish when the light intensity is reduced. Throughout all movements, the right and left pupils remain equal. Even when only one eye is exposed to light while its fellow-eye remains in darkness, the pupillary movements of the stimulated eye and the consensually reacting opposite eye are exactly alike.

As soon as the light is turned off, the pupils redilate (third line of Figure 8). They become as large, or nearly as large as they had been prior to the light stimulus, and the oscillations disappear.

It is obvious that we could not hope to detect the inextensive, slow pupillary reactions to low-intensity light stimuli in the presence of such vigorous background activity. In our studies on the influence of light adaptation upon the pupillary threshold we therefore ran our experiments as follows:

We pre-adapted the eye for 1.5 or two minutes to bright light, then turned the adapting light off and began to stimulate with intermittent low-intensity white or red light stimuli as soon as possible, usually within the first five to seven seconds in darkness.

Figure 9 illustrates such an experiment. In the three test series A, B and C, the eye had been pre-adapted for 1.5 minutes to bright white light (100 percent intensity).

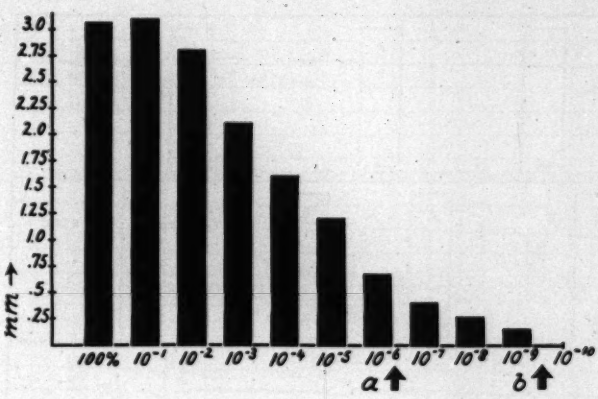

Fig. 7 (Lowenstein and Loewenfeld). AVERAGE EXTENT OF PUPILLARY REFLEXES ELICITED BY WHITE LIGHT STIMULI OF DECREASING INTENSITY.

The height of the columns indicates the average extent of reactions obtained from five normal subjects, 17 to 34 years of age.

The abscissa shows the intensity of the one-second light stimuli, graded in logarithmic steps by Wratten grey filters (see method). One hundred reactions per subject were used for each stimulus intensity. The arrow *a* shows the threshold for color vision ($10^{-4.1}$ to $10^{-4.2}$), the arrow *b* the visual threshold for whight light stimuli ($10^{-9.4}$ to $10^{-9.5}$).

When the intensity of the light was reduced to 1/1,000,000,000th of the full brightness, the pupillary contractions decreased to 1/18th of the original extent.

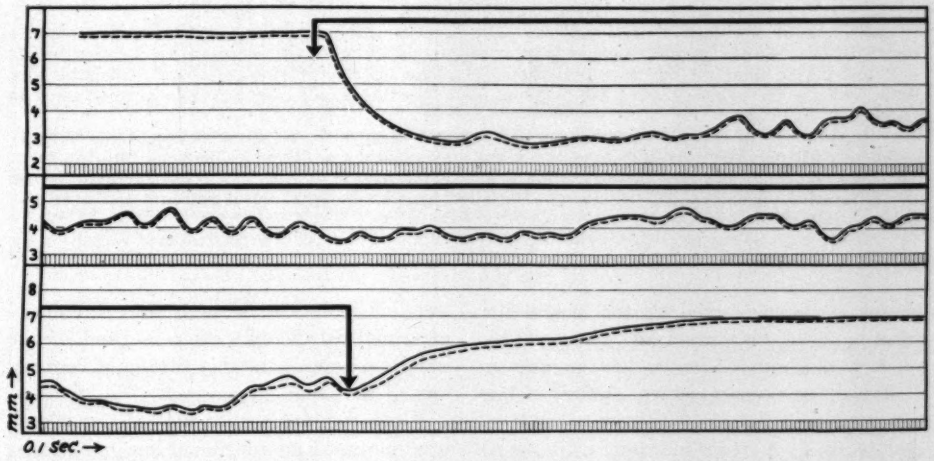

Fig. 8 (Lowenstein and Loewenfeld). PUPILLARY OSCILLATIONS DURING UNILATERAL ADAPTATION TO A BRIGHT, STEADY LIGHT.

Pupillogram of a normal, 24-year-old woman. Pupillary diameter is plotted as the ordinate (in mm) against time as the abscissa (in 0.1 second units). The solid line represents the right, the broken line the left pupil.

First line. The pupils were large and quiet in darkness. When the right eye was exposed to a steady, bright light (20 percent of full intensity, marked by arrow), both pupils contracted, then redilated somewhat and began to oscillate.

Second line. Pupillary oscillations after the right eye had been exposed for three minutes to the same light.

Third line. When the light was turned off (arrow), the pupils dilated and the oscillations disappeared.

The movements of the right and left pupils remained equal throughout the experiment, even though the right eye alone was stimulated while the left eye remained in darkness.

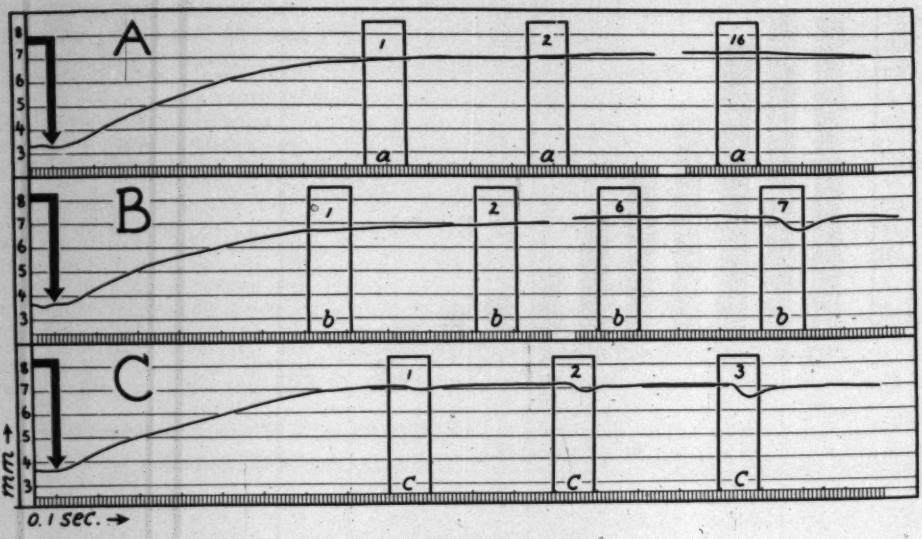

Fig. 9 (Lowenstein and Loewenfeld). PUPILLARY REACTIONS TO LOW-INTENSITY LIGHT STIMULI AFTER PREADAPTATION TO BRIGHT LIGHT.

Pupillogram of a normal, 34-year-old man. The diameter of the consensually reacting left pupil is plotted as the ordinate (in mm.) against time as the abscissa (in 0.1 second units). In the experiments A, B and C, the right eye had been pre-adapted to bright light (100% intensity for 1.5 minute). The arrows indicate the moments when the adapting light was turned off. The pupil dilated and ceased to oscillate (cf. Figure 8).

A. Presentation, at 4-second intervals, of red test stimuli of 1 second duration and of the intensity needed to elicit pupillary threshold reactions in the dark-adapted eye ($10^{-5.7}$). The pupil failed to react.

B. Presentation of red test stimuli one log unit brighter than in A ($10^{-4.7}$). While the first six test stimuli failed to elicit pupillary reactions, a threshold contraction appeared in response to the seventh stimulus.

C. Presentation of red test stimuli two log units brighter than in A. The pupil reacted slightly to the first test stimulus, and as time progressed, the extent of the contractions increased.

At the moment marked by the arrow, the adapting light was turned off. The pupil dilated in darkness. After a few seconds in darkness, one-second red test stimuli of low intensity were presented in four-second cycles. In series A, red test stimuli of the same intensity were used which had elicited pupillary threshold reactions in the dark-adapted eye (intensity $10^{-5.7}$).

After light adaptation, these test stimuli could not be seen by the subject, and they failed to elicit pupillary contractions.

In the second test series (B), the intensity of the red test stimuli was raised 1 log unit ($10^{-4.7}$). Again, the three first test stimuli could not be seen and pupillary reactions failed to appear. The fourth to sixth light stimuli were seen but no pupillary reactions were elicited. The seventh test stimulus—presented 30 seconds after the end of the adapting light—caused a threshold contraction of the pupil.

When the intensity of the red test stimuli was increased an additional log unit (series C), the reactions were not suppressed. Even the first test stimulus was easily seen and caused a pupillary threshold response. As time progressed, the extent of the contractions increased.

The results obtained from our five subjects agreed in general, but quantitative differences were observed. It is especially interesting that the loss of retinal sensitivity due to light-adaptation was not related to the visual and pupillary thresholds of the subject. For example, the threshold responses of

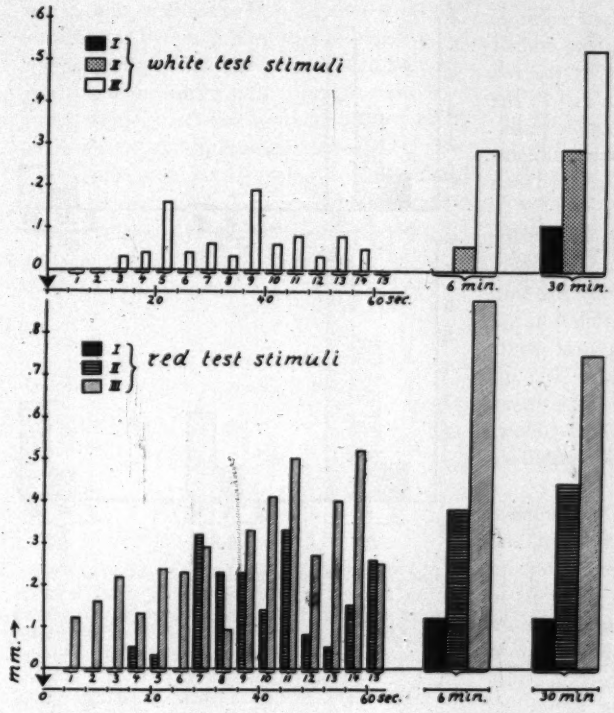

Fig. 10 (Lowenstein and Loewenfeld). EFFECT OF ADAPTATION TO BRIGHT WHITE LIGHT UPON THE PUPILLARY RESPONSES TO WHITE AND TO RED TEST STIMULI OF LOW INTENSITY.

Data derived from the pupillogram of a normal 34-year-old man. The height of the columns indicates the extent of the pupillary contractions elicited by white (top) and by red (bottom) test stimuli of three intensities, namely (I) the light intensity needed to elicit a threshold pupillary reaction of the dark-adapted eye ($10^{-6.3}$ for white, $10^{-5.7}$ for red test stimuli, with visual thresholds of $10^{-9.8}$ for white and $10^{-6.2}$ for red), (II) and (III) one and two log units brighter than (I).

The arrows at the left indicate the moment in each experiment when the bright adapting light (100 percent for 1.5 minutes) was turned off. The slim columns represent the extent of individual reactions to 15 one-second light stimuli which were presented during the first minute in darkness (with 3-second dark-intervals between stimuli). The broader columns represent the average extent of 10 reactions for each stimulus intensity after six and after 30 minutes in darkness.

During the first minute in darkness, the pupil reacted only to the brightest white test stimuli. After six minutes in darkness, the medium-intensity white stimuli elicited feeble contractions, and the reactions to the brightest white stimuli had improved. After 30 minutes of dark-adaptation, the white-light reactions had improved greatly.

In contrast, the red-light test reactions appeared earlier and increased faster. They were fully restored after six minutes in darkness.

subject B, both for white and for red light stimuli, were depressed more profoundly by light adaptation than those of the other subjects, even though subject B had very low thresholds after dark-adaptation. In contrast, the reactions of subject C, with equally low visual and pupillary thresholds after dark-adaptation, were depressed relatively little after light adaptation.

In all experiments, a slightly greater stimulus intensity, or a longer period after the end of the adapting light, were required for pupillary contractions than for visual responses. In all other respects, pupillary and visual reactions agreed completely. When the one was suppressed, the other was suppressed too; when the one was irregular, the other fluctuated also. For any given subject, the findings were consistent when the experiments were repeated.

The experiment of Figure 10 demonstrates the effect of adaptation to bright, white light upon the pupillary reactions to low-intensity red and white test stimuli. In each case, the arrow at the left marks the moment when the white adapting light of 100 percent intensity and 1.5-minute duration was turned off. The height of the columns shows the extent of the pupillary contractions whereby the slim columns at the left represent individual reactions to 15 test stimuli of one-second duration, elicited in four-second cycles during the first minute in darkness. The wide columns at the right

show averages of 10 reactions per column, elicited six and 30 minutes after the end of the adapting light. The intensity of the red and white test stimuli was chosen as (1) the stimulus brightness needed to obtain pupillary threshold reactions after dark-adaptation, (2) one log unit brighter, and (3) an additional log unit brighter.

During the first minute in darkness, the dimmest as well as the medium intensity white test stimuli were not seen by the subject, and pupillary contractions failed to appear. The first two of the brightest white test stimuli—elicited five and nine seconds after the end of the adapting light—were also not seen, while the third and the following stimuli elicited visual and pupillary threshold reactions.

After six minutes in darkness, the dimmest white test stimuli still could not be seen, and the visual responses to the medium-intensity series were irregular. Pupillary reactions to the threshold-intensity stimuli were still absent. The medium intensity stimuli began to elicit feeble and irregular contractions, and the reactions to the brightest test lights had improved but were much less extensive than after complete dark adaptation (30 minutes).

The reactions to red test stimuli were different. During the first minute after the end of the adapting light, the dimmest red test stimuli could not be seen by the subject and no pupillary contraction appeared; but after 6 minutes in darkness, both visual and pupillary functions were completely restored. Visual and pupillary responses to the medium-intensity red test stimuli appeared at the 4th stimulus (18 seconds in darkness), and the reactions to the brightest test series were present immediately. The pupillary contractions increased rapidly in extent and had reached their maximal amplitude after six minutes in darkness.

In Figure 11, the effect of adaptation to bright red light upon the pupillary threshold is compared with the effect of adaptation to bright white light. The white and red test light intensities were the same as in the experiment of Figure 10. As in all these experiments, the red test-stimuli were somewhat more effective than the white test stimuli, and the red-light reactions were less suppressed by light-adaptation than the

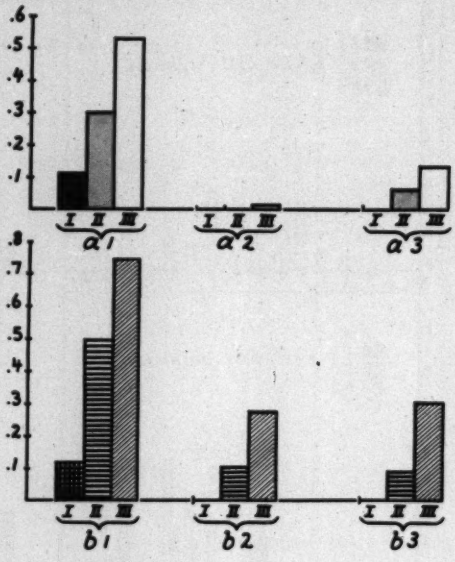

Fig. 11 (Lowenstein and Loewenfeld). EFFECT OF ADAPTATION TO BRIGHT WHITE LIGHT AND TO BRIGHT RED LIGHT UPON PUPILLARY RESPONSES TO WHITE AND TO RED TEST STIMULI OF LOW INTENSITY.

Data derived from the same subject as in Figure 10. In each experiment, the eye was first exposed to bright light for 1.5 minutes (adapting light), then remained in darkness for one minute.

The height of the columns represents average extent of 10 consecutive one-second light stimuli of low intensity, with dark intervals of three seconds between stimuli. The test light intensities were chosen as:

I. The intensity needed to elicit pupillary threshold reactions in the dark-adapted eye.

II and III. One and two log units respectively brighter than (I).

a 1 and b 1. Extent of contractions elicited in the fully dark-adapted eye by the three test-intensities for white (*a*) and for red (*b*).

a 2 and b 2. After adaptation to bright white light, the white-light reactions were almost completely abolished (*a 2*); the red-light reactions were less suppressed (*b 2*).

a 3 and b3. After adaptation to bright red light, the white-light reactions were more extensive than they had been after adaptation to white light. In contrast, the red-light reactions were equally suppressed by adaptation to white and to red light.

white-light reactions. But they were *equally suppressed* by adaptation to white and to red light (compare b 2 and b 3). In contrast, the reactions to white test stimuli recovered much sooner after red-light adaptation (a 3) than after white-light adaptation (a 2).

We conclude that the pupillary threshold reactions are suppressed by light-adaptation and re-appear during dark-adaptation in exactly the same manner as vision. In the dark-adapted eye, the pupillary threshold is much lower for white than for red test-stimuli. After light-adaptation, the reactions to white light stimuli of low intensity are suppressed more profoundly, and they require a much longer time to be restored, than the reactions to red test-stimuli. For both white and red light, the pupillary reactions re-appear sooner and their extent increases more rapidly when the test stimuli are brighter than when they are dimmer.

635 West 165th Street (32).

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Discussion

Dr. Arthur Linksz (New York): I am pleased to be permitted to discuss this paper. I have always been impressed by the work of these distinguished authors, not only by the results they published but also by the prodigious amount of work they put into getting their results.

In the beginning they worked with what now appears to be a primitive method, and it took them thousands and thousands of records and measurements to get the beautiful curves they now get within seconds with their new technique.

I have been asked to discuss two papers today, both of them happen to be based on results achieved with a televisionlike scanning method. Both point toward entirely new possibilities. Now our authors can really start and concentrate on problems rather than measurements. I am sure that this is only the beginning of work we can expect our authors to do in the future.

The essence of the work they are presenting here is that there are two types of pupillary reactions, a rod reaction and a cone reaction. It seems that the rod reaction has a long latency period and is invariable in its form and extent. Cone reaction has a shorter latency; it is at the same time variable, the variations depending on other characteristics of the stimulus.

A few points were not clarified for me by this presentation. Of course it was much too short, and perhaps I can ask a question or two.

I don't quite know what instrument and method they used to determine the visual threshold—whether they applied general illumination of the eyeground; whether retinal periphery or center was tested; whether the patient fixated, and so on. These are important details, since dark-adaptation thresholds depend on the type of the stimulus, the locus of the stimulus, and so on.

The second point I am not quite sure about is the actual state of adaptation. I remember that in one of the tracings four or five stimuli were seen as not working, whereas the sixth or seventh stimulus did cause a pupillary reaction. That means that even a short amount of time was sufficient to change the level of adaptation.

How do the authors know, then, at what level of adaptation they were actually working? Is the intermittence of the light—two or four minutes—sufficient to go back to dark adaptation after intensive lights have been used?

As far as I understand, the tracings are actually caused by the variations of the amount of reflected light. The authors used a red scanning light, and as the pupil contracts less of this light enters the eye and more of it is being reflected. I wonder if the difference between a dark iris and a light iris has any influence. Will a dark iris absorb more of the red light and will therefore tracings be different with a light iris?

Another question that I would like to ask is whether the authors have studied color blind subjects or cases with rod or cone deficiency symptoms to make sure that what they are measuring is rod response or cone response. It would be very nice to test a protanope and to see whether the protanope's pupillogram is different from that of the normal just like his ERG is different. Totally color blind subjects should also be studied. It would be interesting to know whether those monochrome effects which the authors have shown us are rod pupillary responses.

I want to congratulate the authors, and am pleased with the privilege to discuss their paper.

Dr. Irene Loewenfeld (closing): I will begin with the question concerning the measurement of

the pupillary reactions, namely: Does a blue iris reflect more of the infared light used for measuring than does a brown iris? The answer is that there are indeed great differences in reflectivity not only of the iris but also of the pupil of differently pigmented eyes. For this very reason we rejected measuring systems which depend upon the amount of infrared light reflected by the patient's eye. Our method measures the time necessary for a small infrared light spot to scan across the largest diameter of the pupil. This time-measurement is independent of the intensity of the reflected light except, for example, in an albino or in cases with dense cataracts, where the contrast between the iris-area and the pupil-area of the patient's eye would not be sufficient to allow a measurement at all. In all other patients, that is, when the contrast between iris and pupil allows scanning to be done at all, the measurement is independent of the intensity of the reflected beam.

The question on visual threshold: We determined the visual threshold of our subjects in a rather primitive manner. We have to emphasize that we are in the beginning of an extensive program. The determinations were done as follows: We simply used the same stimulating light which we used to elicit the pupillary reactions and added gray filters to reduce the intensity to the value (within 0.1 log unit) at which the subject could see from two to eight of each series of 10 intermittent light stimuli. This light intensity was from 0.5 to 1.0 log unit lower than the one needed to elicit threshold pupillary contractions.

I should add that our light stimulus is rather primitive also in other respects. We are using a slightly divergent beam, 20 mm. in diameter at the subject's eye. From the size of the scotoma which is caused by two minutes of adaptation to the highest intensity of this light, we think that the light covers about a five to six degree angle. Until now, however, we have not investigated the question of the retinal area at all and we have not controlled the size of the entrance pupil. We considered these shortcomings when we drew the conclusions of our experiments. The present work was intended only as an outline, to enable us to find the general range of the phenomena under study. Much more detailed work remains to be done.

The difference between the cone-and rod pupillary responses: The threshold intensity light reflexes, whether elicited by red or by white light, look the same. They both have a long latency period. They both have a shallow and short contraction; but if we raise the stimulus intensity one or two log units above the threshold for white light, the reactions retain these characteristics, in contrast to reactions elicited by red light which become faster, more extensive and more long lasting. We don't know whether this prolonging effect is a specific property of the cone receptive system or whether we have the function of a mere addition; the total intensity of red light stimuli 2.0 log units brighter than the visual threshold for red is, of course, quite a bit higher than the total energy of white light stimuli 2.0 log units brighter than the absolute visual threshold. Therefore it may be that if we go two or three log units above the red threshold we may be getting the retinal rods back into the act, and the fact that the pupil is able to prolong the reaction may be due to the additive function of cones and rods. We don't know at the moment how to circumvent this difficulty in the case of normal subjects. An ideal way of circumventing it was suggested by Dr. Linksz, namely the use of patients with color blindness. We have not yet examined such patients, mainly because we had no such patients available, but we shall do so whenever the opportunity arises.

We want to thank Dr. Linksz for kindly discussing our paper.

PHOTOSENSITIZATION OF PROTEINS BY METHYLENE BLUE*

I. Dehydrogenase activity in rabbit lenses and ciliary body after irradiation in presence of methylene blue

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The recognition of the photosensitizing activity of methylene blue dates back to the pioneer studies of Reitz, 1908,[1] who first reported that methylene blue and visible light were effectively bactericidal when used conjointly. Separately they had no observable effect on bacterial survival. The alteration of sensitivity to light as a result of exposure in the presence of methylene blue was shown to be a general effect observable with widely different biological material,[2, 3] the skin of

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mammals, organisms at all levels of biological organization, isolated enzymes and even individual compounds including certain amino acids.

Before radiant energy becomes immediately available for chemical reaction an aggregate of atoms which can be elevated to an activated state[4] must absorb a quantum of the light. However, the reverse is not true; every quantum of light absorbed does not necessarily result in a photochemical reaction. In complex mixtures, such as methylene blue saturated tissue, although light of all wavelengths is likely to be absorbed, only light absorbed by the chromophore will produce the photosensitized response.

That methylene blue is the chromophore in those complex systems to which it has been added is inferred because a solution of the dye when interposed after the light source reduces or even eliminates the photosensitization. Also methylene blue has two absorption maxima in the visible region of the spectrum, at 610 and 670 mμ and light absorbed by either maximum will produce the photodynamic effect.[5]

The addition of methylene blue to a protein solution results in the formation of a dye-protein complex. For human globin the dissociation constant of this complex was determined[6] to be $9.2 \pm 0.6 \times 10^{-4}$ and for T5 bacteriophage[7] the complex at pH 8.0 was calculated to be 1.03×10^{-5} which increased to 3.23×10^{-5} at pH 7.0. The precise type of complex formed between methylene blue and a given protein has not been determined but the cationic nature of the dye probably directs it toward the anionic centers of the protein. Such an ionic bond complex would not be expected to change very much the absorption spectrum of an ionized chromophore. This is confirmed with methylene blue because the changes in its spectrum upon mixing with protein solutions[5, 6] corresponds closely to changes noted when the concentration of a solution of the dye itself is changed. Further evidence for dye-protein complex formation can be inferred from the data for the effect of pH changes on the photodynamic action of methylene blue[5] and also because competitive inhibition is observed[7] when a photodynamically inactive dye of similar structure is used simultaneously. The fact that the activity of methylene blue as a photosensitizer at different hydrogen ion concentrations parallels the change in the dissociation constant with pH and also parallels the development of anionic character in the protein and cationic character in the dye resulting from changes to higher pH values[5–10] suggests that the dye-protein complex is the active chromophore. However, even though dye-protein complex formation is clearly involved in this effect, it is still not clear and the available data do not allow one to decide if such a complex is formed before or after light activation of a molecule of methylene blue.

The temperature coefficient, Q_{10}, for methylene blue photosensitized inactivation of bacteria,[11, 12] inactivation of bacteriophage[9] and oxidation of a purified protein[8] was found to be greater than one over the physiologically important temperature range. Unless the dissociation constant of the dye-protein complex decreases as the temperature increases a temperature coefficient greater than one indicates that temperature dependant thermal reactions accompany or follow the photochemical reaction.

Quantitative studies of changes in the concentration of methylene blue during irradiation lead to the conclusion that the dye is apparently not used up during the reaction.[2] However, it was proposed and demonstrated that oxygen was utilized during the photodynamic effect[2] with methylene blue and this effect is often referred to as photo-oxidation. Studies of the products resulting from irradiation of methylene blue solutions alone have recently appeared. These findings[13] indicate that the light activated methylene blue molecule is capable of initiating and concluding a series of dehydrogenation reactions. However, these products do not cause the observed biological changes because many investigators have shown that preirradiated methylene blue solutions do not

have the same effect that is observed when the biological material is irradiated with the dye present.

The biological changes which result from methylene blue photo-oxidation are easily discernible but merely reflect the more subtle changes which have occurred in the cellular material itself. When investigators turned their attention to certain enzyme systems they found that along with oxygen uptake during photo-oxidation there occurred a corresponding loss of enzymic activity.[14] It was also found that after irradiation in the presence of methylene blue for sufficient time to inactivate the purified enzyme, the histidine and trytophan residues of the protein were no longer detectable in full amount. Further treatment causes more destruction of these and other aromatic amino acids and eventually even cystine was destroyed. However, the more rapid alteration of these amino acid residues is not limited just to certain enzymes. Apparently complex proteins[6,8] follow the same reaction course. An earlier hypothesis[2] that peptides were produced during photo-oxidation was not observed but an increase in non-protein nitrogen[14] was observed which suggests that ammonia from the heterocyclic rings of histidine and tryptophan was released to the media during the photo-oxidation. Irradiation of a solution containing methylene blue and deoxyribonucleic acid[15] results in a decrease in viscosity of the solution. This indicates that the end result of damage to the deoxyribonucleic acid molecule is depolymerization even if the primary event is dehydrogenation.

The importance of the findings briefly reviewed above stems from the widespread use of methylene blue to provide an alternate pathway or shunt for the cytochrome oxidase system during studies of dehydrogenase activity. If care is not taken to exclude all light adventitious damage to proteins will result when the dye is used for such studies. In studies aimed at understanding the important factors and metabolic processes which provide energy for the sodium pump mechanism apparently operating at or near the lens surface and which maintains optical clarity of the lens, Harris and Gruber[16] used methylene blue as a metachromatic and uncoupling agent. However they found that in the presence of 5×10^{-5}M methylene blue there was complete loss of percent recovery from the cold induced shift in cation balance if the lenses were irradiated during the recovery period. Other work cited above would make it seem evident that under these conditions there should be protein damage and inactivation of enzymes of the lens during the irradiation period. This study was initiated in part to provide a direct proof of such changes and also to probe for information on the enzyme systems which might be important for maintenance of lens transparency.

The purified enzymes which other investigators have inactivated with methylene blue[3] are not as directly linked to energy production in the known metabolic pathways as are the dehydrogenases. It is generally believed that three ATP molecules are produced through linked reactions for each pair of hydrogens removed from a given metabolite. ATP is thought to provide the major source of energy[17] which drives the endergonic reactions important in metabolic and physiologic processes. From these considerations it became apparent that the effect of photosensitization on enzymes closer to the energy rich metabolic reactions must be studied. The dehydrogenases provide a group of enzymes which, because they will for the most part link with reduction of DPN, TPN or certain dyes, could indicate the functional state of the energy source which may be needed for the operation of a pump mechanism.

The histochemical method for the demonstration of succinic dehydrogenase using Nitro-BT[18] was adapted to make it suitable for demonstration of dehydrogenase activity of the intact excised rabbit lens.

Method of irradiation

Lenses used in these studies were for the most part excised from orbs enucleated at the rabbit abattoir immediately after decapi-

tation and were transported to the laboratory in normal saline. Although the excision and other manipulations were done as quickly as possible it was approximately four to five hr. after enucleation before irradiation began. The globes were cut transversely on the posterior sclera with the two incisions roughly at right angles. The quadrants of sclera were peeled back, the lens freed of vitreous body and then excised by circumferential section of the ciliary body. The lens was then transferred with a lens loop to the fluid in which it was incubated and irradiated. In general, each lens was placed with the anterior surface up in 3.0 ml. of the fluid in a 25 by 100 mm. test tube. Before irradiation the stoppered tubes and contents were kept in a covered box packed with ice.

The media in which lenses were preincubated and irradiated has been described previously.[16] In general the modified Tyrodes solution without glucose was used for irradiation studies. During irradiation the tubes were thermostated in a shallow aluminum refrigerator tray into which tap water was directed through a hose in such a manner that the bath was stirred gently. The overflow ran into the drain trough. A 150 W. reflector spot lamp, 55 cm. from the tray, irradiated the specimens. The test tubes holding the lenses rested near the rims on the sides of the tray. The tubes were watched during the irradiation and rotated occasionally to equalize the amount of radiation each lens received during a given period. After irradiation the lens was picked up on a lens loop and washed with a gentle stream of normal saline. It was then placed in the Nitro-BT solution for development of dehydrogenase activity.

Method for dehydrogenase activity

The histochemical method of Nachlas, et al.[18] for localization of succinic dehydrogenase activity in frozen tissue sections was modified slightly to make it suitable for use on excised intact rabbit lenses. A solution containing 1 mg. of 2,2′-di-p-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene) dietrazolium chloride (Nitro-BT)* per ml. of distilled water was diluted just before use with 0.2 M PO_4 buffer, pH 7.45, or with the same buffer containing 0.01 M succinate. The buffered succinate solution was adjusted to pH 7.5. Equal volumes of the two solutions were used which usually was 1.0 to 2.0 ml. and after a lens was added it was incubated 30 min. at 37°C. The tube containing the Nitro-BT solution and lens was occasionally rotated gently to provide uniform access to the solution. After incubation the lens was removed to a 10-percent formalin fixative which stopped the reaction. Although the dark blue colors of the formazan produced during the reaction seemed to deepen a little on standing over night, there was little apparent further change in color after several days in the laboratory even without special precautions being taken.

Within 24 to 48 hours after development of color the lenses were examined individually under the stereomicroscope and notes were made of the location and extent of color relative to the controls. In some of the later experiments and in an effort to provide a numerical index to the necessarily subjective observations of the extent of color development, color photographs were made of the group of lenses of a given experiment. Optical density measurements† were made of the color developed in a given lens and of the control lens both on the same photograph. The lenses were then embedded in paraffin and sectioned, alternate sections being stained with hemotoxylin and eosin.

Results

There is a most striking difference noted between the rate of formazan formation in the freshly excised lens as compared to the

* Obtained from Dajac Laboratories, Philadelphia, Pennsylvania, and it was used without further purification.

† A Photovolt Model 52C paper chromatograph densitometer was adapted for the measurements by masking with a tape covered microscope slide so the illuminated slit was shorter than the width of the lenses on the photograph. Color photographs were made by the Photographic Department of the School of Medical Sciences.

Fig. 1 (Fowlks). Sectioned rabbit lens, ×50, with attached ciliary body. Color developed in intact lens with succinate—Nitro-BT after 40 hrs. incubation in modified Tyrodes medium with glucose and 5×10^{-5}M. methylene blue. Fixed in 10-percent formalin for two days, embedded in paraffin, sectioned and mounted.

ciliary body. The ciliary body begins to darken immediately upon immersion in the 37°C. Nitro-BT solution but the lens darkens slowly with very little blue formazan being formed during the first five minutes of incubation. This indicates that the Nitro-BT must diffuse through the capsule before the insoluble blue formazan is formed. Microscopic examination of the lens sections confirms this supposition since no blue is found in the capsule itself.

Upon gross examination after fixation in formalin it is observed that the attached ciliary body is most darkly stained while the posterior surface of the lens is most lightly stained. There appears to be much more color deposition in the region of the equator and indeed the deposition of color on the anterior surface of the lens shades off to less intensity at the pole. The posterior surface is more uniformly colored from equator to pole.

When examined under the stereo dissecting microscope the lens surface is found to be uniformly stained with the gradation of color from equator to pole noted above more easily discernible. In addition it is noted that the zonules are not visibly colored. There is also a difference between the anterior and posterior suture. The former appears as a white irregular line on a dark blue field while the latter appears as a darkly stained double row of cells.

As can be seen from Figure 1, the deposition of formazan is limited to a thin band immediately subcapsular at the surface of the lens body. Formazan is deposited most densely at the anterior surface. Under ×430 magnification the blue appears most dense at or near the nuclear bodies although it is also found throughout the cytoplasm. The posterior surface has formazan deposition rather uniformly distributed throughout the cytoplasm. A preparation* made by incubation with succinate Nitro-BT at pH 8.0 of fresh frozen sections of a freshly enucleated rabbit orb stained rather uniformly throughout the body of the lens including the nucleus (fig. 3) and the subcapsular band of blue observed in our preparations is almost absent at the posterior surface and greatly reduced at the anterior surface. In this preparation the ciliary body is more uniformly

* I am indebted to Dr. Lee W. Wattenberg of the Department of Pathology for the preparation and Nitro-BT development of these globe sections.

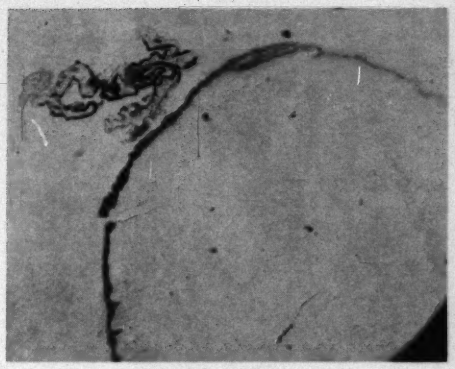

Fig. 2 (Fowlks). Alternate section from lens shown in Figure 1 counterstained with hematoxylin and eosin.

stained throughout than was observed on slides prepared from lenses treated with succinate-Nitro-BT before sectioning.

Lenses treated substantially the same gave closely similar amounts of formazan blue as judged by visual inspection. The 30-minute incubation period provided sufficient time so that a virtual maximum of color was deposited. Even after 20 minutes incubation little additional color appeared during the next 10 minutes unless the amount of dehydrogenase was low. During the initial studies use was made of a buffered succinate-Nitro-BT solution of 0.1 M in succinate to develop dehydrogenase activity. When controls were run without succinate it was found that more color was produced in the controls. It was subsequently found that the endogenous substrate present in the lens itself gave maximum color with Nitro-BT alone in 0.1 M phosphate buffer, pH 7.5. Long incubation at 0°C. in modified Tyrodes solution without glucose gave less color, particularly in the ciliary body, after phosphate-Nitro-BT treatment while development in succinate-Nitro-BT gave normal color development. Up to six hours incubation at 37° in modified Tyrodes without glucose gave no detectable reduction in the dehydrogenase activity of freshly excised lenses when the dehydrogenase activity was developed with the succinate-Nitro-BT solution.

Pre-incubation up to 40 hours at 0°C. and for shorter periods at 20° to 25°C. or 37°C. with 5×10^{-5}M methylene blue added to

Fig. 3 (Fowlks). Fresh frozen section of rabbit globe, ×50, color developed with succinate—Nitro-BT after sectioning.

Fig. 4 (Fowlks). Similar to Figure 1, ×50, except excised lens was illuminated six hours at 22°C. with 1×10^{-5}M methylene blue in the medium but no glucose.

modified Tyrodes media with or without glucose gave only slight differences in color formation on development with succinate-Nitro-BT when compared with controls without methylene blue which had been similarly treated. The slight differences one observes are mainly due to the differences in hue between the two sets. Those lenses without methylene blue produce a formazan which has a reddish tint while those preincubated with methylene blue have more of a cobalt blue color. Nachlas, et al.[18] report no significant difference in color development upon addition of methylene blue to their media. These differences reported here may be due to the slight staining of the lens in the presence of methylene blue alone.

Cyanide added to the phosphate-Nitro-BT development solution had no detectable effect on color development but the addition of 0.025 M malonate to the phosphate-Nitro-BT developer reduced formazan formation to approximately 60 percent compared to a control incubated with succinate-Nitro-BT

Fig. 5 (Fowlks). Alternate section of lens shown in Figure 4 counterstained with hematoxylin and eosin.

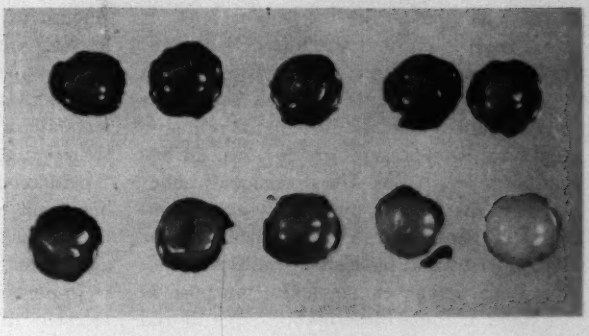

Fig. 6 (Fowlks). Photograph of a series of rabbit lenses which were placed in modified Tyrodes medium without glucose but with 5×10^{-5}M methylene blue. The top row are controls. Lenses in the bottom row were: reading from left to right, (a) irradiated 1.5 hr., (b) irradiated 3.0 hr., (c) irradiated 4.5 hr., (d) irradiated 6.0 hr., (e) incubated 6.0 hr. with methylene blue but not with the phosphate—Nitro-BT solution used for the other lenses.

as measured on a color photograph with the densitometer.

Irradiation of lenses in modified Tyrodes without glucose but with 5×10^{-5}M methylene blue present results in a relatively rapid loss of dehydrogenase activity (fig. 6) of the ciliary body and a slower loss of similar activity in the lens epithelium. Optical density measurements were made on a color photograph of a series of lenses irradiated 1.5, 3.0, 4.5 and 6.0 hours with 5×10^{-5}M methylene blue in Tyrodes without glucose, pH 7.5. The fraction of the optical density of the controls was calculated for each irradiated lens. A plot of these data against the time of irradiation was best fitted with a straight line (fig. 7). Irradiation of lenses without methylene blue or incubation of lenses in the dark with methylene blue does not result in any consistent change in the amount of color produced upon incubation with Nitro-BT developer.

However, it was noted that the presence of glucose in the modified Tyrodes media containing 5×10^{-5}M methylene blue at pH 7.45 seems to protect the lens somewhat from methylene blue photosensitized inhibition of dehydrogenase activity. A more marked protection was observed when the lenses were irradiated in 0.1 M phosphate buffer, pH 7.45.

TABLE 1

ANALYSIS OF RESULTS

Irradiation Medium* With 5×10^{-5}M Methylene Blue	% of Maximum Color of Controls
Modified Tyrodes without glucose	35%†
Modified Tyrodes with glucose	53%
Phosphate buffer 0.1 M	86%

* Irradiated six hours at 22–25°C. and developed with phosphate—Nitro-BT developer.

† Calculated from measurements of optical density of the lens image on a color photograph using a blue filter.

DISCUSSION

A conclusion regarding the particular dehydrogenase which participates in the reactions leading to reduction of Nitro-BT to an insoluble blue diformazan by the excised intact rabbit lens cannot be made on the basis of the experiments so far performed. A number of different dehydrogenases have been re-

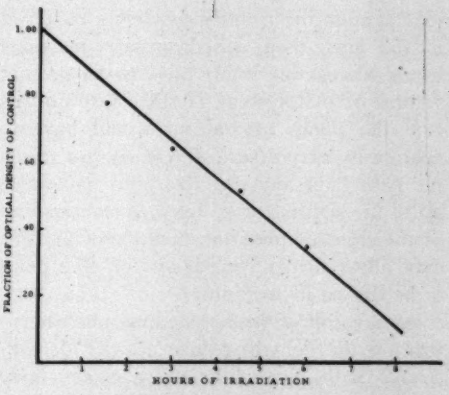

Fig. 7 (Fowlks). Relative dehydrogenase activity of excised rabbit lenses incubated with 5×10^{-5}M methylene blue and irradiated at 27°C. 55 cm. from 150 W. reflector light. Data from lenses shown in Figure 6.

ported[19] as present in the rabbit lens. It would seem likely that succinic dehydrogenase has some activity in these lenses just on the basis of the results reported here. It is quite evident that other systems or reducing agents are also present which cause the reduction of Nitro-BT. The presence of endogeneous substrates in the freshly excised intact rabbit lens makes rather difficult a clear cut demonstration by histochemical methods of any particular enzyme. Our purpose at this stage of these investigations was to find a method which was suitable for demonstrating changes in dehydrogenase activity resulting from the photodynamic effect of methylene blue. The method reported here shows promise of fulfilling that purpose.

On the list[3] of specific enzymes which have been altered by the photodynamic action of methylene blue, dehydrogenases as a group appear to be absent. It might be argued that since succinic dehydrogenase has been shown to be readily inactivated by other photosensitizers[20] methylene blue photosensitization would also inactivate it and perhaps other dehydrogenases as well. Indeed it was on the basis of this argument that the presently reported work was started. If the hypothesis is correct, that a system is operating at or near the lens surface capable of producing sufficient energy to pump ions against the osmotic gradients observed in the lens, then dehydrogenase reactions would almost inevitably have to be present as part of that system. If such were not the case the pump mechanism would have to operate by way of a different energy transfer path than any that has been uncovered up to the present time. Further examination of the effect of photosensitization of the lens may allow firmer conclusions on this point to be drawn in the future.

As a result of methylene blue photosensitization the dehydrogenase activity demonstrable with Nitro-BT in the ciliary body falls sharply. The dehydrogenase activity found in the lens epithelium and at the posterior surface disappear during the illumination procedure directly proportional to the amount of illumination; a result entirely in keeping with previous studies on the photodynamic effect of methylene blue. Microscopic examination of sections of the lens and ciliary body does not reveal gross destruction of tissue as a result of the photosensitized damage to dehydrogenase activity, but what effect if any photo-oxidation has on capsular or cellular permeability must await further investigation.

Important from the viewpoint of those interested in the mechanism of photosensitization is the finding that glucose protects lens dehydrogenase when present during illumination. This suggests that there is a reduction of dye-protein complex formation if the active centers of an enzyme is occupied by substrate molecules.

Summary

1. A brief review of methylene blue photosensitization has been presented.

2. The method for localization of succinic dehydrogenase activity using Nitro-BT has been adapted for use with the intact excised rabbit lens with attached ciliary body. Most dehydrogenase activity was found in the ciliary body. Pigment formed in the lens was greatest in the epithelium although the posterior surface was also lightly stained.

3. Preliminary studies indicate that Nitro-BT reduction to the blue diformazan pigment may occur as a result of reducing reactions in addition to those linked to succinate dehydrogenation.

4. The dehydrogenase activity of ciliary body is rapidly destroyed upon illumination with visible light when rabbit lenses excised by ciliary body resection are incubated in 5×10^{-5}M methylene blue. Dehydrogenase activity of the lens is also destroyed under these conditions and is proportional to the duration of illumination.

5. Some protection of dehydrogenase activity is obtained when glucose or inorganic phosphate are present in the media during illumination.

A-684 Mayo Memorial Hospital (14).

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Discussion

Zacharias Dische, M.D. (New York): The experimental data of Dr. Fowlks appear interesting as photosensitization of the lens has been in certain cases suspected for along time as a possible etiological factor in cataract formation. Dr. Fowlks' experiments seemed to demonstrate that at least one of the points of attack is dehydrogenases, which are mainly localized in the equatorial anterior portion of the lens and the posterior lens capsule. The latter finding again stresses the possible significance of metabolic processes in the lens capsule for the pump mechanisms of the lens.

The fact that the sensitizing effect was shown to represent a photo-oxidation and that glucose has a significant inhibitory effect on the sensitation, appear to be in good agreement with the results of the studies on the metabolism of the lens which stress the importance of the glucose-6-phosphate dehydrogenase for the lenticular metabolism.

The protection against sensitation observed in presence of phosphate also appears interesting insofar as experiments in our laboratory have shown that phosphate at concentrations at which, according to Fowlks, it inhibits photosensitization by methylene blue, has a strongly inhibitory effect also on the enzymes involved in the pentose phosphate metabolism. We know that this reaction sequence is a complementary pathway which, together with glucose-6-phosphate dehydrogenase activity is an essential part of the direct oxidative pathway of glucose.

The fact that this inhibition can be observed at a concentration of 0.1 M, is in itself not irreconcilable with a pathological significance of these desensitizing effects of phosphates, as theoretically in certain strategic points inside the cells, much higher concentrations of ions can be assumed than correspond to the average concentration calculated for the whole tissue.

The fact that the photosensitization by methylene blue involves a catalysis of oxidative processes, raises a question whether in the case of the lens this photosensitization does effect not only enzymes but the structural proteins of the lens as well which, as we know, contain easily oxidizable thiolic groups. Such oxidation can induce significant structural changes in lens fibers.

Dr. David Cogan (Boston): I have not had an

opportunity to read Dr. Fowlks' paper and I did not understand from his presentation the role of the methylene blue and dehydrogenases.

A suitable dehydrogenase will mediate a transfer of electrons from succinate to tetrazolium on incubation. Isn't methylene blue acting by short-circuiting the dehydrogenase system rather than acting on it?

In other words, I wonder whether methylene blue isn't acting as an electron transfer intermediate, just as is the dehydrogenase? Are we justified in inferring that this has anything to do with dehydrogenases.

Secondly, Dr. Fowlks said the cyanide did not lessen the tetrazolium reduction. I wonder whether it *enhanced* it. After all, the tetrazolium is competing with the cytochrome system for the hydrogen, and one would expect, a priori, that cyanide might actually enhance the tetrazolium reduction since it inhibits the cytochrome activity.

DR. W. L. FOWLKS (closing): I would like to start with Dr. Cogan's remarks. I believe the evidence is quite clear that we are dealing with enzymic dehydrogenation in this case. The tetrazolium salt used was Nitro-BT which is not reduced at all by succinate and methylene blue in the times used for incubations in these experiments and, according to the authors who originally reported[18] Nitro-BT, the extent of reaction is not affected by the presence of methylene blue. Nitro-BT does not react in quite the same way that some of the other tetrazolium salts do. Also, the data indicate clearly that low concentrations of malonate and high concentrations of succinate inhibit the reaction which would be true for an enzymic process but not for direct chemical reaction.

In my original presentation, I had meant to emphasize, and I apologize for not doing so, that *no* increase in activity results from the presence of cyanide. The purpose of using cyanide, of course, was to poison the cytochrome oxidase system, but, since Nitro-BT shows maximum effectiveness even in the presence of oxygen,[18] it by-passes the cytochrome oxidase step and thus would not be affected favorably by cyanide.

With regard to the theory that sulfhydryl groups are lost during methylene blue photosensitization we have an old and venerable theory that, like many others, has not stood up under the scrutiny of careful investigation. Recent literature concerned with methylene blue photosensitization of specific proteins contains clear cut evidence that the sulfur containing amino acids are less sensitive to photo-oxidative conditions that are histidine and tryptophan. When the sulfur-containing amino acids, cystine, cysteine, and methionine, are compared individually with the individual aromatic amino acids they are found to be destroyed with equal or greater facility under identical conditions. However, the situation is quite different when the sulfur-containing amino acids are included in the peptide chain of a specific protein. In the peptide chain, they show unusual resistance to photo-oxidation and the available evidence suggests that histidine then tryptophan are preferentially destroyed and indeed SH groups may appear during subsequent photo-oxidation due in part to an unsymmetric oxidation of the disulfide linkage of cystine.

OCULAR CHANGES INDUCED BY POLYSACCHARIDES*

III. PAPER CHROMATOGRAPHIC FRACTIONATION OF A BIOLOGICALLY ACTIVE HYALURONIC ACID SULFATE PREPARATION

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AND
JOHN E. HARRIS, M.D.
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Previous work in this laboratory has demonstrated that a sulfated hyaluronic acid derivative† resembling heparin rather closely in chemical composition and in many biologic properties differs conspicuously from heparin in at least one important respect; that is, when injected into the eye, the sulfated hyaluronic acid produces severe ocular pathology, while heparin exerts only mild and transitory effects, if any.[1-3] This contrast between the actions of the two acid mucopolysaccharides on the eye is the sole qualitative difference in their biologic prop-

* From the John E. Weeks Memorial Laboratory, Department of Ophthalmology, University of Oregon Medical School and the Department of Ophthalmology, University of Minnesota School of Medicine. Supported by Grant No. B-1978 of the National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Maryland.

† Prepared by sulfating hyaluronic acid from umbilical cord under mild conditions. Supplied through the courtesy of Dr. Robert L. Craig of G. D. Searle and Company.

erties thus far identified. Balazs, Hogberg, and Laurent,[4] who prepared and studied a similar sulfated hyaluronic acid derivative, observed marked differences in the potencies of their product and of heparin when the two were assayed for various types of biologic activity, but both products displayed the same general spectrum of biologic properties.

Use of this pathogenic polysaccharide in conjunction with heparin provides an excellent experimental situation for well-controlled studies of the physiologic and metabolic changes associated with various ocular disorders. In pursuing such investigations, however, it was important first to determine how long the acid mucopolysaccharides remained in the eye in their original forms. Therefore, some of their physiocochemical properties were compared in an effort to devise an unambiguous method for identifying them in biologic material. Such studies served to emphasize further the similarities between the hyaluronic acid sulfate and heparin but failed to afford a means for distinguishing between them. Thus, it was found that the hyaluronic acid sulfate cannot be unequivocally distinguished from heparin either by paper electrophoresis or by spectrophotometric study of their toluidine blue complexes.[3]

In the course of the paper electrophoretic studies, it was observed that the distribution of acid mucopolysaccharides on the strips bearing the hyaluronic acid sulfate did not appear to be that of a completely homogeneous material.[3] This observation, in turn, raised a question regarding the nature of the factor (s) responsible for the pathogenic activity of the material and necessitated further investigation of the composition of the preparation. Additional evidence that the hyaluronic acid sulfate may not be entirely homogeneous was obtained when the material was subjected to paper chromatography.

Molho and Molho-Lacroix[5] had reported the paper chromatographic separation of a commercial heparin preparation into two components, one which migrated and one which remained at the origin, using a n-propanol:water solvent system. Kerby,[6] on the other hand, had found heparin to migrate as a single spot when solvent composed of 37-percent n-propanol in a phosphate buffer was used.

On paper chromatography in either of these two solvents, the hyaluronic acid sulfate is resolved into two principal components, one which migrates at essentially the same rate as a commercial heparin and one which remains at the point of application. Comparison of the chemical, physical, and biologic properties of these two main fractions with those of the parent preparation was then indicated.

A small supply of each was isolated using the n-propanol:water solvent system, both components of which are completely volatile, with a thick paper in a manner similar to that outlined by Weissman, Meyer, Sampson, and Linker[7] for the isolation of oligosaccharides derived from hyaluronic acid. After eluting the polysaccharide fractions from the paper with distilled water and lyophilozing the resulting solutions to dryness, some of the properties of the products obtained were compared with those of the parent material and of heparin.

Physicochemical properties studied included paper chromatographic and paper electrophoretic behavior, ultraviolet absorption spectra and absorption spectra of their toluidine blue complexes. Since all the biologic properties of the sulfated hyaluronic acid preparations thus far studied, except that of producing ocular disorders, differ from those of heparin quantitatively rather than qualitatively, it seemed desirable to investigate the two fractions of the hyaluronic acid sulfate for possible gradations in potency in some test of biologic activity other than the production of ocular disorders.

Some of the tests previously applied to such preparations require the application of rather specialized techniques, but antocoagulant activities can be compared rather easily.

Therefore, the anticoagulant properties of the chromatographic fractions, as well as their capacities to produce ophthalmic disease, were examined. When these studies had been completed, some of the remaining mobile material was analyzed chemically. The limited supply of the stationary component was exhausted, however, making it necessary to postpone analysis of that product.

Methods

Chromatographic fractionation of the hyaluronic acid sulfate

The n-propanol:water system of Molho and Molho-Lacroix,[5] consisting of 1.0 volume of n-propanol plus 1.5 volumes of distilled water, was used for this purpose. These workers failed to indicate whether the ascending or descending technique was employed, but Kerby[6] specified the ascending technique for 72 hours at room temperature. Preliminary experiments showed, however, that a satisfactory separation of the hyaluronic acid sulfate into its component parts could be achieved in only 18 hours if the descending technique was employed. Accordingly, the latter technique, carried out at room temperature, was adopted for these studies.

For preparative purposes, 0.10 ml. of a 10 mg./ml. (that is, a 1.0 mg. sample) solution of the hyaluronic acid sulfate in distilled water was applied to a thick paper (Whatman No. 3 mm.) in a uniform streak across the paper[7] along a line located so as to fall 1.5 cm. below the support rod of a Fisher strip-paper chromatographic apparatus. The solution was applied slowly, with intermittent drying, keeping the streak as narrow as possible (always less than a centimeter in width). Dimensions of the chromatographic strips were 2 by 22.5 inches and the apparatus used accommodated three such strips, stapled together, on each side. At the end of eighteen hours, the strips were removed from the tank and dried in air. When dry, one strip from each group of six was stained with toluidine blue (fig. 1) to locate the acid mucopolysaccharide (metachromatic) material and sections of the unstained strips corresponding to the mobile and stationary components were cut out, eluted with distilled water, and lyophilized to dryness. In a few experiments, some of the metachromatic material lying between the two main components and the orthochromatic material appearing at the solvent front were eluted and concentrated by lyophilization to obtain a solution for spectrophotometric study.

Studies of chromatographic fractions

1. Paper chromatography

The two components isolated as described above were rechromatographed in the same solvent and also in the solvent developed by Kerby[6] composed of 37-percent n-propanol in pH 6.5, M/15 phosphate buffer. For these experiments, 5.0 µl. aliquots of 3.0 mg./ml. (15 µg. total sample) solutions of the two fractions of the hyaluronic acid sulfate, the hyaluronic acid sulfate itself, and heparin were used. Hyaluronic acid, prepared in a concentration of 6.0 mg./ml. was also applied in a 5.0 µl. aliquot (30 µg. total sample).

Earlier work had shown[3] that the commercial sample of hyaluronic acid (biologic source unspecified) used in these studies did not stain with toluidine blue, but did stain

Fig. 1 (Talman and Harris). Preparative paper chromatogram, showing separation of the sulfated hyaluronic acid into two principal components. Solvent: n-propanol:water (1:1.5.) Toluidine blue stain.

with another acid mucopolysaccharide stain, alcian blue. Therefore, a number of chromatograms were run in duplicate, one being stained with toluidine blue and the other with alcian blue, not only to detect hyaluronic acid, if present, but also to avoid overlooking some acid mucopolysaccharide component exhibiting a similar discrepancy in staining properties. Staining with alcian blue was carried out as previously described.[3] Staining with toluidine blue was carried out essentially as described by Leitner and Kerby[8] except that fixation in alcohol and ether was found to be unnecessary and three five-minute washes in two-percent acetic acid after staining were found to reduce the background color of the paper considerably more than a single two-percent acetic acid wash followed by a rinse in running tap water.

2. Paper electrophoresis

The paper electrophoretic mobilities of the two chromatographic fractions were compared with those of the parent material and of heparin by the technique previously described.[3]

3. Spectrophotometric studies

a. *Ultraviolet absorption spectra.* Ultraviolet absorption spectra were run on solutions contains 2.0 mg./ml. of heparin, 3.0 mg./ml. of the hyaluronic acid sulfate and 0.375 mg./ml. of the stationary and mobile components of the hyaluronic acid sulfate. The metachromatic mucopolysaccharide material moving at an intermediate rate chromatogrophically and the orthochromatic material migrating with the solvent front were present in such small quantities that lyophilization of these material to dryness and subsequently redissolving them was impractical. Therefore, spectral studies of these fractions were carried out on solutions of unknown concentration obtained by eluting the appropriate sections of the paper with distilled water and concentrating the resulting solutions by lyophilization.

b. *Absorption spectra of toluidine blue complexes.* These complexes were prepared as previously described.[3] A 1.0 ml. aliquot of the solution of unknown concentration of the acid mucopolysaccharide material moving at an intermediate rate was treated in a similar manner.

4. Anticoagulant activity

The amount of heparin usually recommended for anticoagulant purposes is 0.1 mg./ml. of blood. Therefore, the effect of this amount of hyaluronic acid sulfate, and of each of its two main components, on the coagulation time of rabbit blood was investigated. For this purpose, each material was dissolved in 0.85-percent NaCl in a concentration of 1.0 mg./ml. and 0.10 ml. of each solution was transferred to two dry serology tubes. Control tubes contained 0.10 ml. of 0.85% NaCl. After adding 1.0 ml. of freshly drawn rabbit blood, a modified (two-tube) Lee and White clotting time determination was carried out. All materials were tested with blood from the same rabbit and seven animals were used in all.

5. Pathogenic effects in the eye

Several rabbits received intraocular injections of each of the chromatographic fractions of the hyaluronic acid sulfate, and of the original hyaluronic acid sulfate itself, in the sites and amounts outlined below. The concentrations of all these materials were adjusted so that the amount administered was contained in a volume of 0.05 ml.

Site of Injection: Anterior chamber

5 rabbits
O.D. 5.0 mgs. Hyaluronic acid sulfate.
O.S. 5.0 mgs. Mobile component, hyaluronic acid sulfate.

6 rabbits
O.D. 1.0 mg. Hyaluronic acid sulfate.
O.S. 1.0 mg. Mobile component, hyaluronic acid sulfate.

3 rabbits
O.D. 2.3 mgs. Hyaluronic acid sulfate.
O.S. 2.3 mgs. Stationary component, hyaluronic acid sulfate.

Site of injection: Vitreous

3 rabbits
O.D. 5.0 mgs. of Hyaluronic acid sulfate on each of two successive days.

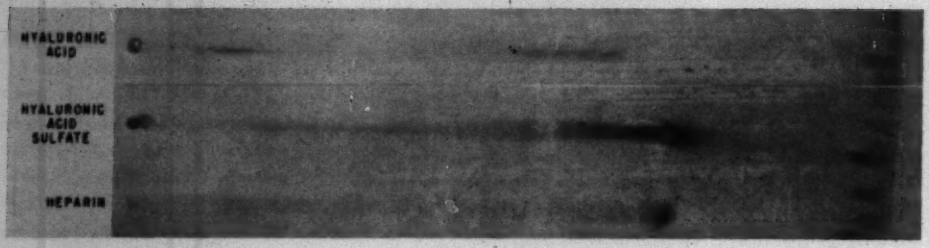

Fig. 2 (Talman and Harris). Paper chromatogram comparing the behavior of the sulfated hyaluronic acid with that of hyaluronic acid and heparin. Solvent: n-propanol: water (1:1.5). Alcian blue stain.

O.S. 5.0 mgs. of Mobile component, hyaluronic acid sulfate on each of two successive days.

After seven days, rabbits injected via the anterior chamber were killed and their corneas excised for further study. The two corneas from one animal were prepared for histologic examination and those from the remaining animals for determinations of their water contents. Rabbits injected intravitreally were sacrificed after two weeks and their eyes removed and sectioned.

6. Chemical analysis*

Results

Paper chromatography

The paper chromatographic behavior of the parent hyaluronic acid sulfate, of heparin, and of a commercial sample of hyaluronic acid in the n-propanol:water solvent system is illustrated in Figure 2. There, it can be seen that the hyaluronic acid sulfate separates into two main components, one comprising roughly seven percent of the original material, which remains at the origin and another, comprising 90 percent or more of the original material, which migrates at about the same rate as heparin. The balance of the material appears as a streak between the two main components extending not quite back to the starting line. All of the heparin migrated as a single spot in this particular experiment, and this was usually the case when less than 10 μg. of heparin were used. The use of larger amounts to intensify the spots for photographic purposes often led to streaking. A large portion of the hyaluronic acid remained at the point of application. Although two light streaks of acid mucopolysaccharide are detectable further down the paper, neither of the latter traveled as far as heparin or the mobile component of the hyaluronic acid sulfate. The stationary component of the hyaluronic acid sulfate is probably not unsulfated hyaluronic acid, however.

The sample of hyaluronic acid used in these studies does not stain metachromatically with toluidine blue while the stationary fraction of the hyaluronic acid sulfate is strongly metachromatic, and also, as may be seen below, the stationary component migrates faster on paper electrophoresis than either this sample of hyaluronic acid[8] or one, which was metachromatic, studied by Rienits.[9] Except that the hyaluronic acid was stained by alcian blue but not by toluidine blue, results with the two stains were the same, although toluidine appears to be slightly more sensitive.

Results of rechromatographing the two fractions in the same solvent system are shown in Figure 3. The material isolated from the starting line of the preparative paper chromatograms again appears at that point with minimal streaking a short distance down the paper. The mobile component exhibits a well-defined spot migrating the same distance as heparin and part of the parent material, but some metachromatic material also remained at the origin.

* Analysis was performed by Huffman Microanalytical Laboratories, Wheatridge, Colorado.

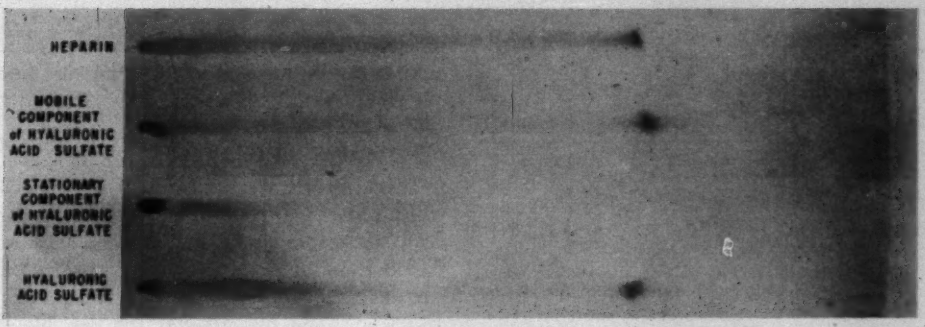

Fig. 3 (Talman and Harris). Paper chromatogram comparing the behavior of the two main components of the sulfated hyaluronic acid with that of the unfractionated material and of heparin. Solvent: n-propanol: water (1:1.5). Toluidine blue stain.

As indicated by Figure 4, this phenomenon appears to be largely the result of overloading in order to improve the photographic qualities of the chromatograms. When smaller quantities of material were applied, little residual material appeared at the origin. This was especially noticeable with the n-propanol: phosphate buffer solvent. Results with the stationary component are shown in Figure 5. With the n-propanol: water system, that is, the solvent used for isolating the material, it remained principally at the origin with minimal streaking. With the n-propanol: phosphate buffer system, a streak of metachromatic material was observed which extended, at the furthest, less than half as far down the paper as the mobile component of the parent material but the great majority of the fraction remained at the starting line.

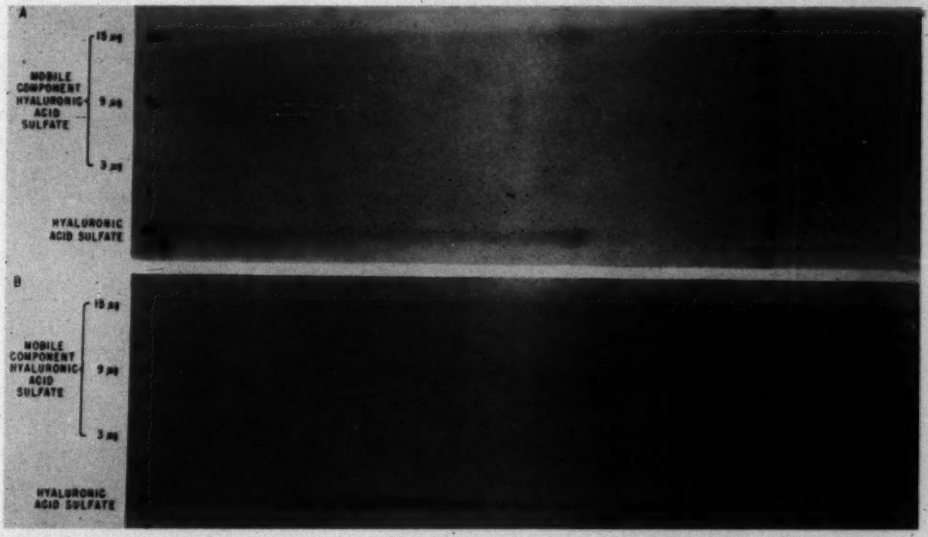

Fig. 4 (Talman and Harris). Paper chromatograms obtained using increasing quantities of the mobile component of the sulfated hyaluronic acid with two solvent systems. (A) n-propanol: water (1:1.5). (B) 37-percent n-propanol in pH 6.5, M/15 phosphate buffer. Toluidine blue stain.

TABLE 1

PAPER ELECTROPHORESIS OF ACID MUCOPOLYSACCHARIDES IN 0.1 M PHOSPHATE BUFFER, pH 6.2

Acid Mucopolysaccharide	Band Limits*
Hyaluronic acid sulfate	6.0–7.1
Mobile component hyaluronic acid sulfate	6.1–6.8
Stationary component hyaluronic acid sulfate	5.5–6.5
Heparin	5.8–7.1

* Distance (in cm.) of trailing and leading edges, respectively, of main band from point of application.

PAPER ELECTROPHORESIS

These results are summarized in Table 1. In this experiment, the hyaluronic acid sulfate and heparin behaved almost identically. Of the fractions derived from the hyaluronic acid sulfate, the mobile component migrated at nearly the same rate as the bulk of the parent material, but was localized in a slightly narrower band than the latter. The stationary component did not appear in an appreciably narrower band than the parent material, but moved somewhat more slowly. On the paper electrophoretic strips, the stationary component appeared to be somewhat more strongly metachromatic than the mobile component.

SPECTROPHOTOMETRIC STUDIES

These results are summarized in Table 2 which shows that heparin and the hyaluronic acid sulfate preparation differ somewhat in the location of their ultraviolet absorption maxima, the former absorbing maximally at 270 mμ. and the latter at 261 mμ. None of the fractions into which the hyaluronic acid sulfate was separated chromatographically exhibited an ultraviolet absorption maximum. These findings correspond with those of Molho and Molho-Lacroix[5] who found the 270 mμ. absorption maximum of their commercial heparin to be absent in the material which migrated paper chromatographically.

Spectrophotometric study of the toluidine blue complexes of the various fractions provided further evidence that they differ from

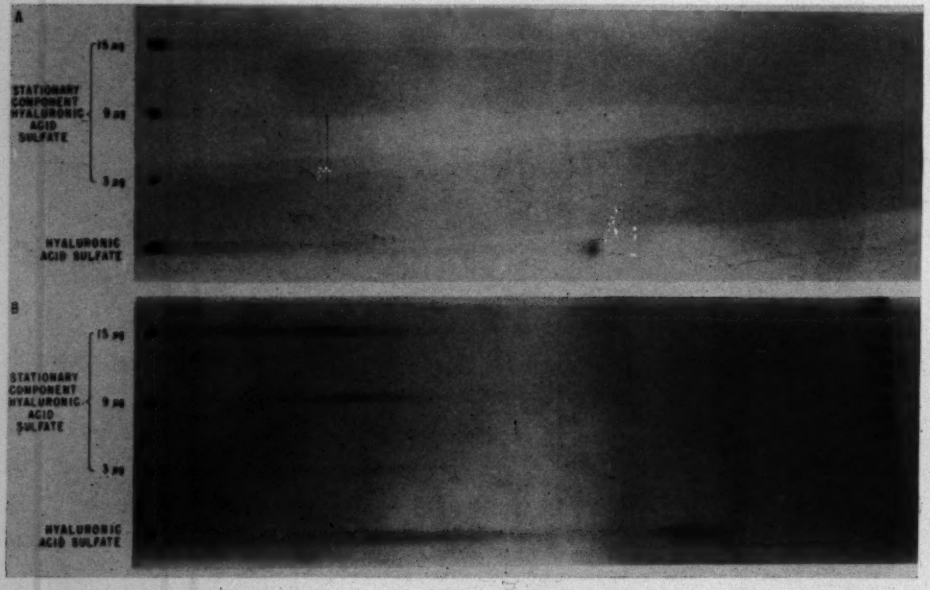

Fig. 5 (Talman and Harris). Paper chromatograms obtained using increasing quantities of the stationary component of the sulfated hyaluronic acid with two solvent systems. (A) n-propanol:water (1:1.5). (B) 37-percent n-propanol in pH 6.5 M/15 phosphate buffer. Toluidine blue stain.

TABLE 2

SPECTROPHOTOMETRIC ABSORPTION MAXIMA OF VARIOUS ACID MUCOPOLYSACCHARIDES AND OF THEIR COMPLEXES WITH TOLUIDINE BLUE

Acid Mucopolysaccharide	Absorption Maximum	
	Ultra-violet	Toluidine Blue Complex
Heparin	270	531–536
Hyaluronic acid sulfate	261	534–535
Mobile component hyaluronic acid sulfate	None	542
Stationary component hyaluronic acid sulfate	None	542–543
Intermediate material hyaluronic acid sulfate	None	530–531
Orthochromatic material at solvent front	None	—
Toluidine blue	290	640

the original. Thus, although the absorption maxima of the toluidine blue complexes of both the stationary and mobile components are located at almost exactly the same position, this maximum appeared at an appreciably higher wavelength than that of the parent material. The absorption maximum of the complex of the intermediate material, which absorbs light very strongly, appeared at a slightly lower wavelength than that of the parent preparation and at a considerably lower wavelength (10-11 mμ. lower) than the two main fractions.

ANTICOAGULANT ACTIVITY

Both fractions, as well as the parent material, exhibit marked anticoagulant activity, but the stationary component is a much more potent anticoagulant than the other two. Blood treated with the stationary fraction was fluid and completely unclotted after two hours. With the mobile fraction, small clots, or a fragile clot which allowed the tube to be inverted despite the presence of fluid blood in the bottom of the tube, were noted after two hours. Blood treated with the parent material was unclotted, but extremely viscous at the end of two hours.

PATHOGENIC EFFECTS ON THE EYE

Both chromatographic fractions of the hyaluronic acid sulfate retain the property of producing ocular disorders. Injecting 1.0 mg. of the mobile material into the anterior chamber had no effect, but 5.0 mg. caused marked hydration of the cornea within 24 hours. The contralateral eyes, which received 5.0 mg. of the parent material via the same route, showed only slightly injected irises and possibly slight corneal hydration along the needle track.

TABLE 3

HYDRATION OF THE CORNEA IN VIVO SEVEN DAYS AFTER INJECTING THE HYALURONIC ACID SULFATE OR THE MOBILE COMPONENT OF THE HYALURONIC ACID SULFATE INTO THE ANTERIOR CHAMBER

Procedure	Rabbit No.	Water Content % of Normal
5 mg. (0.05 cc.) hyaluronic acid sulfate into anterior chamber of right eye	1	106
	2	109
	3	137
	6	113
	13	129
	14	119
	15	201
	16	112
	17	120
	18	105
	28	95
	29	95
	30	90
		Range: 90–201
		Average: 118
5 mg. (0.05 cc.) mobile component of hyaluronic acid sulfate into anterior chamber of left eye	1	168
	3	173
	4	150
	6	310
		Range: 150–310
		Average: 200

Corneal hydrations, seven days after treatment, of eyes injected with the two drugs are listed in Table 3. In general, the mobile component produced greater corneal hydration than the original material, although, as usually observed in experiments of this type, the results were quite variable. From a statistical viewpoint, a larger series of animals treated in this way would be desirable. However, close examination of the data presented in Table 3 shows only one instance (rabbit No. 15) in which the water content of the cornea from an eye treated with the original material overlaps the values found for eyes treated with the mobile component despite the fact that figures are given for a considerably larger number of animals treated with the

original material. Furthermore, comparison of the results on the two eyes of rabbits 1, 3, and 6 shows corneal hydration to be uniformly greater in the eye receiving the mobile component.

Section of the two corneas from one of the rabbits from this group revealed conspicuous histologic changes in the eye receiving the mobile component and only minor changes in the eye injected with the original preparation. The cornea from the eye treated with the mobile component showed marked vascularization throughout the entire stroma and a thickening and cellular modification of the endothelial layer was also observed (fig. 6). The absence of conspicious changes in the eye receiving a single injection of the unfractionated hyaluronic acid sulfate in the

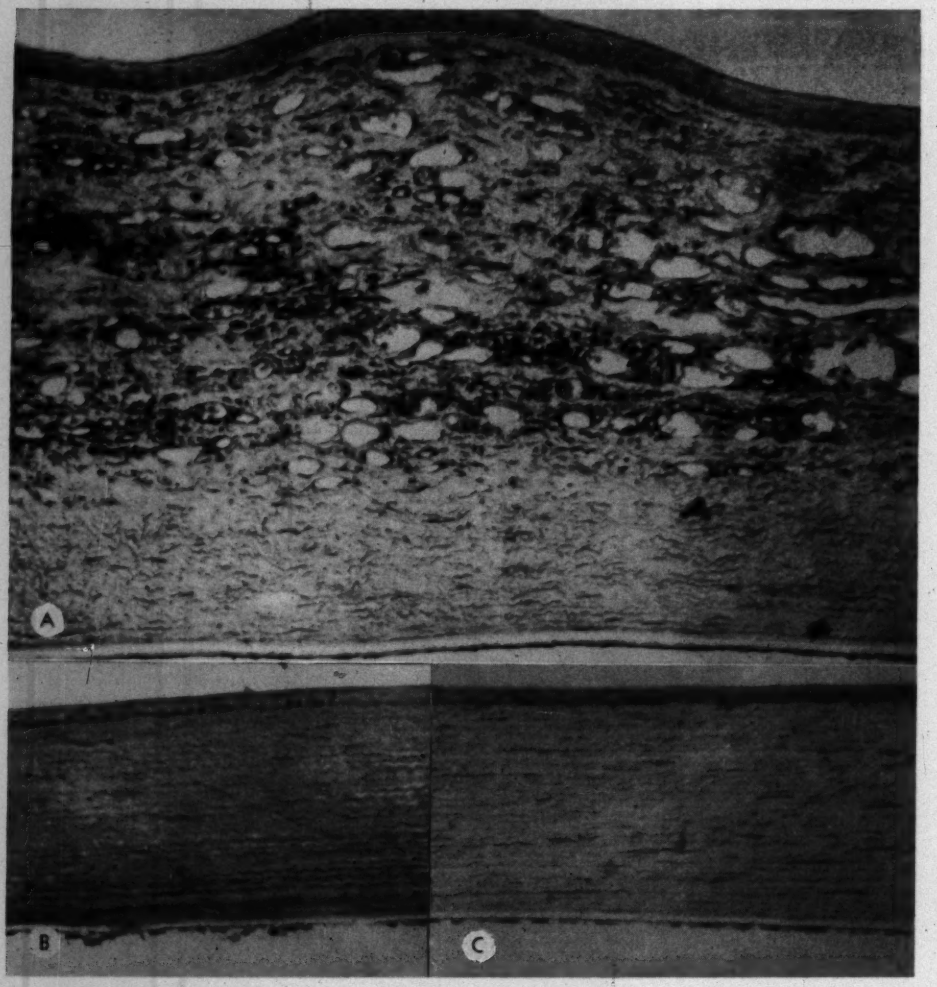

Fig. 6 (Talman and Harris). Histologic sections of rabbit corneas from eyes injected via the anterior chamber with: (A) 5.0 mg. of the mobile component of the hyaluronic acid sulfate. (B) 5.0 mg. of the unfractionated hyaluronic acid sulfate. (C) Untreated cornea.

anterior chamber conforms with the previous observations that two injections via this route on successive days were required to produce permanent pathologic changes.[2]

When the two materials were injected into the vitreous, the eyes receiving the parent material developed subepithelial or diffuse corneal opacities, with thickening of the cornea in some cases. Intraocular hemorrhage occurred at least once in all eyes injected intravitreally with the mobile component. A second hemorrhage occurred in two instances and two cases eventually developed subepithelial corneal opacities.

Twenty-four hours after injecting 2.3 mg. of the stationary component via the anterior chamber, marked injection of the iris and varying degrees of corneal hydration were noted in all eyes. After three days the corneal hydration began to subside in two eyes, but the third became progressively worse. A tendency toward corneal hydration is usually noted for a few days after injecting heparin via the anterior chamber and in this respect, the ocular response to a small dose of the stationary component resembled that to heparin in two of three eyes. The appearance of definite pathology in the third eye after this single small dose of the material suggests that its action is probably not entirely similar to that of heparin, however.

Chemical analysis

Analyses of the parent hyaluronic acid sulfate and of the mobile component (table 4), particularly the S:N ratio, indicate that the mobile component may contain two less sulfate groups per disaccharide unit than the original material.

TABLE 4

Chemical analysis of the original hyaluronic acid sulfate preparation and of the mobile material isolated from it

	Hyaluronic Acid Sulfate (%)	Mobile Component Hyaluronic Acid Sulfate (%)
Moisture	8.9	10.45
Carbon	23.37	20.93
Hydrogen	2.70	2.77
Oxygen	48.45	45.00
Nitrogen	1.97	2.11
Sulfur	13.69	5.26
Residue from C & H	40.12	50.7
S:N Ratio	3.04:1	1.09:1

Comment

Whenever a mixture is resolved into its component parts, one must consider the possibility that the products obtained may have been altered in some way in the isolation procedure. The absence of a maximum from the ultraviolet absorption spectra of any of the chromatographic fractions of the hyaluronic acid sulfate when the parent material displayed a peak at 261 mμ might be construed as evidence for such alteration. However, the biologic, and most of the chemical, properties of these fractions are those which might reasonably be expected if there were no alteration.

Molho and Molho-Lacroix[5] reported a similar phenomenon with heparin. The commercial heparin preparation they used as a starting material exhibited an absorption maximum at 270 mμ, which, they noted, is incompatible with the structure of heparin, and this maximum was absent from the spectrum of their mobile chromatographic fraction which possessed potent anticoagulant activity. Thus, it seems likely that the ultraviolet absorption maxima displayed by heparin and the unfractionated hyaluronic acid sulfate preparation reflect the presence of a contaminant which is eliminated in the chromatographic procedure.

Since absoprtion at 280 mμ is frequently used to assay protein content in enzyme studies, it seems reasonable to suggest that this contaminant may consist of a protein which is loosely bound to the polysaccharides, and that this loose binding may serve to shift the absorption maximum of the protein to a somewhat lower wavelength.

Comparison of the chemical compositions of the parent preparation and the mobile material provides evidence indicating alter-

tion of the latter in the course of the chromatographic separation which is less easily explained. The mobile fraction contains so much less sulfur than the original material and comprises such a large proportion of the latter as to raise serious doubt whether the remaining fractions can be sufficiently rich in sulfur to account for this difference. The most probable change would seem to be the hydrolytic cleavage of two sulfate groups per disaccharide unit, but the product of any such hydrolysis must be stable since the material isolated as the mobile fraction exhibited the same chromatographic behavior after isolation as before.

The possibility that such an hydrolysis may ensue whenever the hyaluronic acid sulfate is dissolved in water is suggested by the close resemblance of the mobile fraction to the parent material in paper electrophoretic behavior. The stationary fraction, on the other hand, appears to be stable. In any event, when that product was rechromatographed, essentially all of it remained at the point of application and there was no evidence for the presence of a material resembling the mobile fraction. Although further study is required to establish definitely whether or not the original material underwent chemical changes during the isolation procedure, the fact remains that the fractions isolated retain the ability to produce ocular pathology.

Although it is tempting to conclude from these studies that the mobile component is more potent in producing ocular pathology than the parent material, these observations must be substantiated and extended before such a conclusion can safely be drawn. Neither do the data thus far obtained permit definite conclusions regarding the relative potencies of the two chromatographic fractions in producing ocular pathology. In their studies comparing a similar sulfated hyaluronic acid preparation with various heparins, Balazs, et al.[4] found antithrombin activity to decrease with increasing sulfur content of the substance examined while antihyaluronidase, anticoagulant and antigrowth (fibroblasts in tissue culture) activities increase with increasing sulfur content. It is not yet possible to decide whether the property of producing ocular disorders falls into either category; but it is interesting to note that our results probably confirm those of Balazs and associates with respect to anticoagulant activity; that is, the stationary component is a more potent anticoagulant and very likely contains more sulfur than the mobile component. Furthermore, it should be pointed out that the relative anticoagulant potencies of these two chromatographic fractions appear to represent another qualitative difference in the properties of the hyaluronic acid sulfate and heparin.

In contrast to the findings outlined here, Molho and Molho-Lacroix[5] reported their heparin fraction which migrated to be about 25 times as active an anticoagulant as the fraction remaining at the origin and that, among the sulfated acid mucopolysaccharides they studied, only those which migrated chromatographically in a manner similar to heparin displayed anticoagulant activity.

Even should some correlation between the sulfur contents of the hyaluronic acid sulfate fractions and their pathogenic potencies upon injection into the eye eventually be established, the observation would offer no clue as to the possible mechanism of action of the unnatural acid mucopolysaccharides. Since heparin produces no permanent pathologic changes when injected into the eye, more subtle differences than total sulfur content must be responsible. Preliminary studies with some of the hyaluronic acid (from umbilical cord) used as a starting material for the preparation of the hyaluronic acid sulfate suggest that the basic carbohydrate structure of the material may play an important role in its effects on ocular tissues. The latter does not negate the previous suggestion[3] that the hyaluronic acid sulfate, being structurally similar to but not identical with heparin, may interfere with some of the functions of heparin, or with

the functions of other natural acid mucopolysaccharides which are unaffected by heparin.

Recent studies of gargoylism, a congenital disorder in which corneal opacities usually constitute part of the syndrome, and of corneal macular dystrophy in adults suggest that nature may have performed some experiments on the effects of acid mucopolysaccharides on the eye long before the artificial sulfation of hyaluronic acid from umbilical cord was undertaken. Thus, Uzman[11] has shown, by isolating it from the livers and spleens of such patients, that the storage substance found in the tissues of patients with gargoylism consists of two chemical entities, one of which is a metachromatic acid mucopolysaccharide.

Subsequently, Dorfman and Lorincz[12] and Meyer and associates[13] detected and identified two acid mucopolysaccharides (chondroitin sulfate B and heparin monosulfate) in the urine of such patients. Also, Jones and Zimmerman[14] have reported the presence of a material having the staining characteristics of an acid mucopolysaccharide in corneal buttons obtained from patients with macular dystrophy.

The appearance of ocular pathology in association with acid mucopolysaccharides under two different circumstances, that is, after the injection of an unnatural acid mucopolysaccharide into the eye and in diseases characterized by the appearance of abnormal, or abnormal amounts of, acid mucopolysaccharides in the tissues and urine, may offer a further clue to the pathogenesis of these ophthalmologic disturbances.

As Forgacs and Franceschetti have pointed out, the syndrome of gargoylism may logically be considered to reflect an error in the metabolism of mucopolysaccharide[15] and the corneal opacities which occur in this condition might arise from such a disturbance in the metabolism of the cornea itself. However, the appearance of corneal opacities as one of the sequelae of injecting a chemically altered acid mucopolysaccharide into the eye suggests the possibility that the corneal disturbance of gargoylism may result from the action on the cornea of abnormal, or abnormal amounts of, acid mucopolysaccharides produced elsewhere in the body.

In any event, the demonstration of abnormal accumulations and excretions of acid mucopolysaccharides in ophthalmic diseases which occur spontaneously makes the appearance of ocular pathology as a consequence of injecting an unnatural acid mucopolysaccharide seem somewhat less remarkable.

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Discussion

Endre A. Balazs (Boston): The biological effect of sulfated hyaluronic acid is an important field about which little is known. I feel that the authors are to be congratulated for their courage in attacking this very difficult and challenging problem. Ten years ago we made a similar attempt, but gave it up.

The main difficulty that the authors encountered was that the sulfated hyaluronic acid which they used was a polydisperse material of unknown purity. Since the hyaluron sulfate used in the experiments described was made from umbilical cord hyaluronic acid, I wonder what the chondroitin sulfate content of this hyaluronic acid preparation was. Depending on the preparation of the tissue and the separation method used in umbilical cord hyaluronic acid preparations, up to 10-percent chondroitin sulfate may be present.

In the work presented there is evidence of protein impurities in both the hyaluron sulfate and the heparin preparations. In commercial heparin preparations we have found quite often, but not always, an ultraviolet-absorbing material which was not dialyzable and which gave an absorption maximum in the region described by the authors.

Further difficulties with regard to the inhomogeneity of the material will occur after sulfation. I don't know which sulfation procedure was used by the authors, but we found, using chlorosulfonic acid in pyridine, that the original high-molecular hyaluronic acid was degraded. The resulting hyaluron sulfate was polydisperse with regard to molecular size and the number of sulfate ester groups per molecule. The variation in the degree of sulfation of the polysaccharide molecules is characteristic not only of synthetic hyaluron sulfate but also of the naturally occurring heparins (Jeanloz: Structure of heparin, Fed. Proc., **17**:1082, 1958).

A further complication can occur in the course of the sulfation of hyaluronic acid; namely, deacetylation of the hexosamine amino group, and possible N-sulfamylation of the same group. In our preparation of hyaluron sulfate there was evidence for deacetylation, but it was not certain whether or not N-sulfamylation also took place. The chemical structure of the heparins has not as yet been sufficiently established to allow one to state with certainty that all amino groups are sulfated and that no N-acetyl group is present. Heparins of lower sulfate ester content (heparin monosulfate) have an N-acetyl group.

The biological activity of hyaluron sulfate or heparin can depend, both qualitatively and quantitatively, on the following chemical structural differences: on the number of sulfate ester groups and their position, on the substituent on the amino group (sulfate or acetyl), on the size of the molecule, and last, but not least, on the hexosaminidic linkage.

The authors correctly point out that by separating two fractions from hyaluron sulfate one isolates only two of the many possible fractions present, each of which may have a different biological activity.

I sympathize with the authors in their concern about the discrepancy between the sulfur-nitrogen ratio of the hyaluron sulfate and its so-called "mobile component," and the amount of the stationary fraction collected on the paper chromatograms. Paper electrophoretic studies apparently offered no solution to this problem. By free electrophoresis one should be able to separate or, at least, to obtain some indication of the presence of various components with different surface charge. Desulfation in the course of paper chromatography is not likely.

It is interesting that a swelling effect on the cornea was observed after injecting this material into the anterior chamber. One wonders, of course, about the apparently nonspecific antienzyme activity of sulfated polysaccharides in general. This toxic effect on the corneal endothelium could result in swelling of the stroma.

REPRESENTATION OF THE NEAR-RESPONSE ON THE CEREBRAL CORTEX OF THE MACAQUE*

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INTRODUCTION

The ability to fixate binocularly, with clarity, stereopsis and variable depth of focus an infinite number of loci in any frontal plane between infinity and a near point is found only in primates and man. This complex visual motor phenomenon is dependent upon the intricate and precise linkage of accommodation, convergence, and pupillary constriction—a triad that has been called the near response, the near reflex, or the near synkinesis.[1] For the purpose of this paper the term near-response is arbitrarily employed. However, this term does not adequately emphasize either the importance or the complexity of this triad. The near-response embodies the most highly developed and complicated visual sensory and motor relationships, and imparts to higher primates and man a capacity for a three-dimensional concept of space not possessed by lesser animals. It is, in essence, the visual motor response characteristic of man. An understanding of the near response is vital to the interpretation of normal and abnormal oculomotor functions. Most concomitant strabismus may be interpreted as an abnormality in this response.

The near response is a phylogenetically late acquisition, and attains its full development only in primates. Certain species with laterally placed eyes and limited binocular vision have monocular pupillary constriction associated with accommodation but lack entirely a convergence mechanism. Convergence appears first in higher primates and man superimposed on a more primitive conjugate gaze mechanism. It is supposed that it is the development of convergence as well as stereopsis that goes hand in hand with the appearance of uncrossed visual nerve fibers in the chiasm.[2,3]

Deductions and theories on the neuroanatomy of the near response are widespread, but are based on relatively few facts. Experimental work presents many problems since it must be performed on primates. It is the purpose of this paper to briefly review some pertinent literature, bring together some known facts, and add some new ones in the hope of increasing our understanding of this phenomenon.

LITERATURE

Review of the literature to date reveals no experiments in which accommodation of the eyes has been produced by stimulation of the cerebral cortex. On the other hand, convergence of the eyes has been occasionally produced by cortical stimulation by four different techniques: (1) simultaneous stimulation in the primate of corresponding areas in the two frontal or the two occipital eye fields,[4] (2) severing of the lateral recti in the primate prior to cortical stimulation in the frontal eye field,[5] (3) unilateral cortical stimulation in the primate and human,[6,7] and (4) cortical stimulation in the cat, in areas known to produce conjugate horizontal deviations, preceded by transection of the brainstem at the junction of the midbrain and pons.[8]

The investigators employing these techniques were interested in the physiology of eye movements in general and convergence was noted only as an uncommon ancillary phenomenon to other findings. Little attempt was made in the experimental protocols to analyze the nature of the convergence movements produced, to describe in detail the brain regions concerned in convergence, or to investigate the neuronal pathways in-

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volved. Russel,[8] in 1894, however, in his experiments did incriminate the most caudal part of the principal fissure of the brain of the monkey. With the Marchi technique the neuronal projections of the frontal and occipital eye fields, from which horizontal conjugate deviations are readily produced, have been traced into the midbrain and pons.[9,10] It is assumed that the innervational patterns subserving convergence are carried by the same or closely related pathways, since in general they are elicited from within or in close proximity to the same eye fields.

Pupillary constriction has rarely resulted from stimulation in the frontal lobe.[11] It has been more readily obtained from stimulation in the preoccipital cortex in the monkey by Schäfer[12] in 1888 and in the cat by Barris[13] in 1936. Barris obtained bilateral pupillary constriction by stimulating the lower end of the most posterior part of the posterior lateral gyrus in the cat. Employing the Marchi technique he traced fibers from that area along the lateral wall of the lateral ventricle, over the lateral geniculate body, and through the stratum zonale of the thalamus into the pretectal area.

Stimulation of the superior colliculi has not resulted in either accommodation, convergence, or pupillary constriction. Ranson and Magoun[14] and their students worked out the neuroanatomic basis for pupillary constriction due to light. Discussion of their work is not relevant to this paper except to emphasize that the integrity of the cerebral cortex is not necessary in order to elicit the pupillary response to light and that the pretectal area contains the supranuclear connections and the synaptic relays for the light reflex.

Accommodation, inward movement of the eye (unilateral adduction), and pupillary constriction have resulted from stimulation of discrete cell masses in the midbrain deep to the superior colliculi. Hensen and Völckers,[15] in 1878, removed the cerebral cortex and large sections of the diencephalon of the dog under morphine narcosis and stimulated the exposed floor of the third ventricle and the iter. Stimulation of the floor of the third ventricle produced accommodation. This was demonstrated by inserting a needle in the equator of the eye up to the surface of the vitreous. Upon faradic stimulation of the brainstem movement of the needle was interpreted as indicating ciliary muscle contraction. Stimulation more caudally within the iter produced iris contraction and, still more caudally, contraction of the homolateral internal rectus (fig. 1).

Bender and Weinstein[16] in 1943 in the monkey employing the Horsely-Clark stereotaxic instrument and minute currents obtained pupillary constriction from stimulating in the dorsal and rostral region of the oculomotor nucleus. The pupillary constriction was usually bilateral but on occasion occurred only in the homolateral eye. Stimulating one mm. below the focus for pupillary constriction resulted in prominent budging of both irises. This bulging was attributed to ciliary muscle contraction. Adduction of the homolateral eye was produced when stimulating more ventrally and caudally. By bringing the electrodes closer to the midline and increasing the current bilateral sharp adduction was produced.

Bender and Weinstein state, without amplification, that this bilateral adduction could be distinguished from the slower bilateral convergence elicited from other regions. Thus, stimulation in three distinct areas in the midbrain produced accommodation manifested by bulging of the irises without pupillary constriction, adduction of the homolateral eye, and pupillary constriction that was frequently homolateral. The small-celled Edinger Westphal nucleus was presumed to contain a rostral-dorsal group of cells subserving the final common path for pupillary constriction and a more ventral-caudal group subserving the final common path for accommodation.

Crosby and Woodburne,[17] in 1943, morphologically distinguished a rostral and caudal Edinger-Westphal nucleus but with no

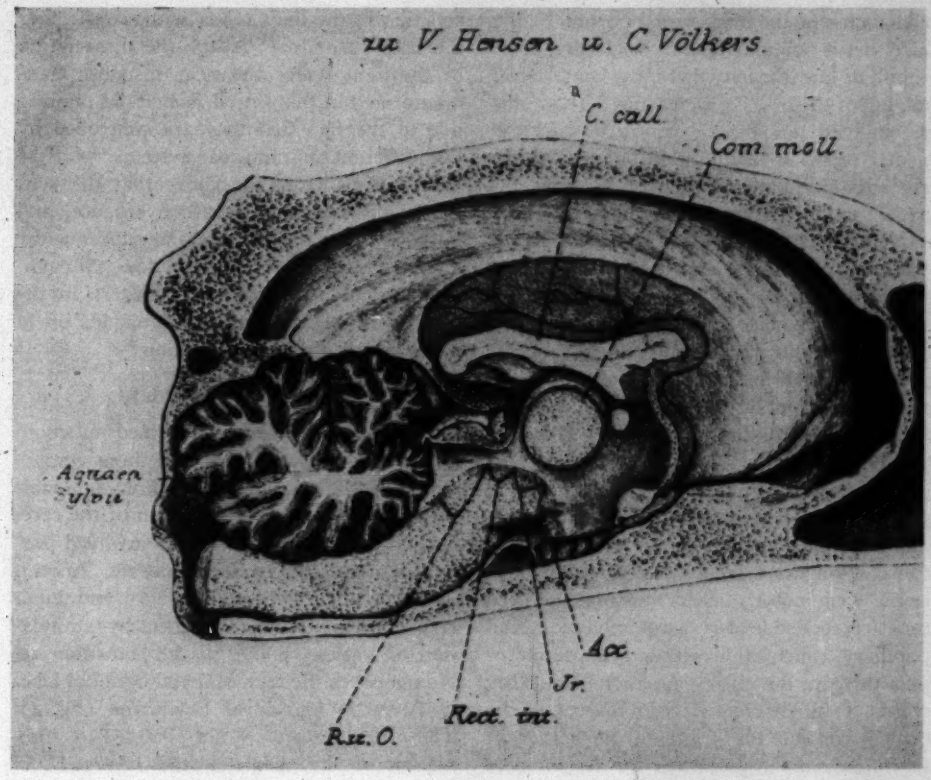

Fig. 1 (Jampel). A reproduction of a picture of historical interest from Hensen and Völkers (1878) illustrating their experimental findings from faradic stimulation in the brainstem of the dog (see text). (Acc., center for accommodation; C. call., corpus callosum; Com. moll., commissura mollis; Ir., center for iris sphincter; Rect. int., center for internal rectus; Ru. O., location of the center for the rest of the eye muscles.)

clear line of demarcation separating them. The rostral nucleus was assumed to be associated with pupillary constriction and the caudal nucleus with accommodation. In the large celled body of the oculomotor nucleus are a group of cells innervating the homolateral medial rectus and probably acting as the final common pathway for all impulses to the medial rectus. The evidence for Brouwer's[18] contention that the medial recti are innervated by two separate and distinct groups of cells, one subserving adduction in conjugate gaze (versions) and the other adduction in convergence (vergence) is controversial.

Investigators have obtained accommodation and pupillary constriction from stimulation of the intracranial portion of the third nerve,[19] the ciliary ganglion,[20] and the posterior ciliary nerves as they enter the globe.[21] The changes in accommodation of the eye may be noted by observing the movement of a needle placed into the choroid at the equator, by photographing the Purkinje images, by retinoscopy, by the use of the coincident optometer, by evoked potentials from the ciliary muscle, and by the observation of bulging of the irises in the absense of pupillary activity.

Lowenstein,[19] in 1956, stimulated the

medial side of the intracranial portion of the third nerve with a bipolar electrode. With stimuli of low intensity there was homolateral bulging of the iris. By increasing the stimulus he obtained homolateral contraction of the medial rectus in addition to bulging of the iris without pupillary activity. Further increase in the stimulus strength produced active homolateral pupillary contraction with vigorous iris bulging and contraction of the medial rectus. By slightly moving the electrode he was able to obtain pupillary contraction without iris bulging and contraction of the internal rectus. By nicotinizing the ciliary ganglion he obtained contraction of the medial rectus but no pupillary constriction or iris bulging.

The evidence just outlined is cited to prove that the impulses that result in accommodation, convergence, and pupillary constriction arise from discrete cell groups in the oculomotor nucleus and that the neurones responsible for accommodation and pupillary constriction either synapse in or pass through the ciliary ganglion.[19] In other words, from discrete nuclear masses in the oculomotor nucleus originate the nerves of the final common pathways for accommodation, pupillary constriction, and unilateral adduction. There are no cell masses within the oculomotor nucleus that appear to act as an integrating or controlling center for convergence or the near response. Warwick,[22] in 1955, presented other experimental and morphological evidence that supports this idea.

There are probably separate cell groups in the oculomotor nucleus for pupillary constriction associated with the light stimulus and for the pupillary constriction associated with the near response and these cells must be in very close proximity. Supporting the idea for separate cell groups is the evidence for a separate peripheral pathway for each function, that is, the presence of accessory autonomic ganglia,[23] and the fact that extirpation of the ciliary ganglion in the chimpanzee has caused loss of pupillary constriction in the light reflex without affecting the near response.[24] Also, the dissociation of the light reflex and near response commonly seen in the Argyll Robertson pupil is due to the fact that they are controlled by two different supranuclear systems.[19]

It follows from the above that the synthesis of accommodation, convergence, and pupillary constriction occurs at supranuclear levels. The question remains does this synthesis occur in hypothetical centers in the brainstem, as frequently postulated, or at other levels of nervous function?

Experimental work

Experimental work performed on monkeys[25] demonstrated that the three components of the near response are obtainable by unilateral faradic stimulation of the cerebral cortex and that the areas involved project anatomically to the midbrain. Accommodation of the eyes, convergence, and pupillary constriction were elicited by stimulation of various points in the transition region between the temporal and occipital lobes in Areas 19 and 22 of Brodmann (fig. 2). These responses were not obtained by stimulation of the striate cortex (Area 17 of Brodmann). It is stressed that the results appeared to rely upon the use of minimal amounts of ether anesthetic and the maintenance of the animal at a level of consciousness not too far below the point where voluntary eye movements are abolished.[26]

Accommodation was qualitatively determined by the use of retinoscopy. The Fincham coincident refractometer requires good fixation of the eyes, and since it is not possible to maintain fixation in the intact experimental animal, it was found unsuitable for determining accommodation in these experiments. Under ether anesthesia the refraction of the eye was determined and, to the correction in the vertical meridian, a minus one spherical lens was algebraically added inducing a distinct "with" motion of the retinoscopic reflex. If the retinoscopic reflex changed from a "with" to an "against" mo-

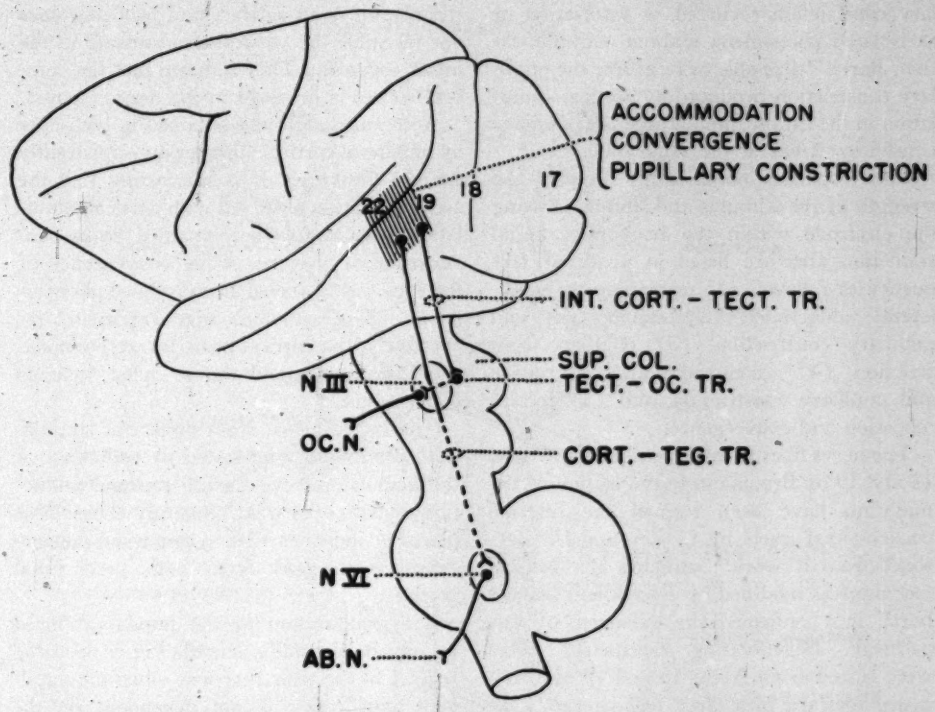

Fig. 2 (Jampel). A diagramatic representation of the cerebral cortex and brainstem of the Macaca mulatta depicting the cortical areas associated with the near response and their neuronal projections into the tectum and tegmentum. (AB. N., abducens nerve; CORT.-TEG. TR., cortico-tegmental tract; INT. CORT.-TECT. TR., internal cortico-tectal tract; NIII., oculomotor nucleus; NVI., abducens nucleus; OC. N., oculomotor nerve; SUP. COL., superior colliculus; TECT.-OC. TR., tecto-oculomotor tract; 17, 18, 19, and 20 are Brodmann's areas.)

tion following stimulation of the cortex, accommodation was assumed to have taken place. No attempt was made to quantify the accommodative changes, and any change of less than one diopter would not have been detected by the technique employed.

When a change from a "with" to an "against" motion in the retinoscopic reflex was obtained by unilateral faradic stimulation in the cortical areas mentioned above (fig. 2) accommodation was assumed to have taken place. The accommodation occurred in both eyes and in each instance was accompanied by convergence movements and, except one occasion, by pupillary constriction.

The convergence movements consisted most frequently of adduction of the homolateral eye. Less frequently a bilateral asymmetric adduction of both eyes was observed in which the greater movement was in the homolateral eye. Also, a contralateral gaze movement was noted in which the homolateral eye moved a greater distance or at a greater velocity than the contralateral eye. This last observation was also made in 1917 by Leyton and Sherrington[6] in the frontal eye field.

The pupillary constriction elicited from unilateral stimulation was usually bilateral and equal. On occasion anisocoria with the larger pupil on the same side was noted following the stimulation. The pupils would constrict no more than one third of their original diameters. Convergence and pupil-

lary constriction occurred in association or as isolated phenomena without accommodation. Barris[13] was able to reinforce the pupillary constriction produced by cortical stimulation in the cat by stimulating both pupillary constrictor areas at the same time.

The responses obtained by varying the strength of the stimulus and slightly shifting the electrode within the temporal-occipital transition area are listed in order of frequency as follows: (1) convergence (homolateral adduction), (2) convergence and pupillary constriction, (3) pupillary constriction, (4) accommodation, convergence and pupillary constriction, and (5) accommodation and convergence.

The nerve fiber pathways which link Areas 18 and 19 of Brodmann to the tectum of the midbrain have been termed the internal cortico-tectal tracts by Crosby and Henderson.[26] Recent work[25] utilizing the Marchi technique as modified by Swank and Davenport[27] has confirmed the existence of this pathway. Degenerating myelinated fibers were traced from Areas 18 and 19 of Brodmann forward in a layer just lateral to the visual radiations and parallel to them as far forward as the pulvinar of the thalamus. From here they turned medially across the internal capsule through the pulvinar to terminate in the tectum of the midbrain (INT. CORT.-TECT. TR., fig. 2 and fig. 3). Other degenerated fibers turned into the tegmentum of the midbrain without reaching the tectal areas (CORT.-TEG. TR., fig. 2 and fig. 3). They could be traced only as far as the level of the inferior colliculi, but are assumed to reach the abducens nucleus. They are probably responsible for the inhibition of the lateral recti that takes place during convergence.

Comment

Accommodation, convergence, and pupillary constriction have resulted from faradic stimulation in overlapping areas of the preoccipital (peristriate) cortex (fig. 2). These results are significant even though electrical stimulation is an artifact and probably does not resemble the cortical mechanisms in the intact organism. They indicate that the cerebral cortex is involved in the near-response.

Accommodation was obtained in both eyes by unilateral cortical stimulation—apparently for the first time. It is interesting that the accommodation observed with cortical stimulation was always accompanied by a convergence of the eyes while convergence of the eyes was observed as an isolated phenomenon. These reactions may represent, respectively, the cortical basis for accommodative-convergence and the so-called fusional convergence.[28]

The convergence from unilateral stimulation was mostly manifested by adduction of the homolateral eye, though unequal adduction of both eyes was frequently noted. This probably indicates that symmetrical convergence is initiated from both preoccipital cortices.

The constriction of the pupils produced by unilateral faradic stimulation is probably related to the near response since the pupillary light reflex is not dependent on the integrity of the cerebral cortex. The constriction was never more than one third of the original pupillary diameter. Barris[13] demonstrated, in his experiments on pupillary constriction in the cat from cerebral stimulation, that bilateral stimulation in corresponding cortical areas caused greater pupillary constriction than unilateral stimulation. Thus, in pupillary constriction associated with the near-response there is probably bilateral initiation and summation.

The proposition of this paper is that the components of the near-response are controlled and synthesized at the highest level of nervous activity, the cerebral cortex, and then are modified at a functionally lower level, the pretectum and tectum of the midbrain. The neuroanatomic scheme is presented in Figure 3.

At the cortical level the initiating of this response, as is true for all complex motor phenomena, is influenced, augmented, and

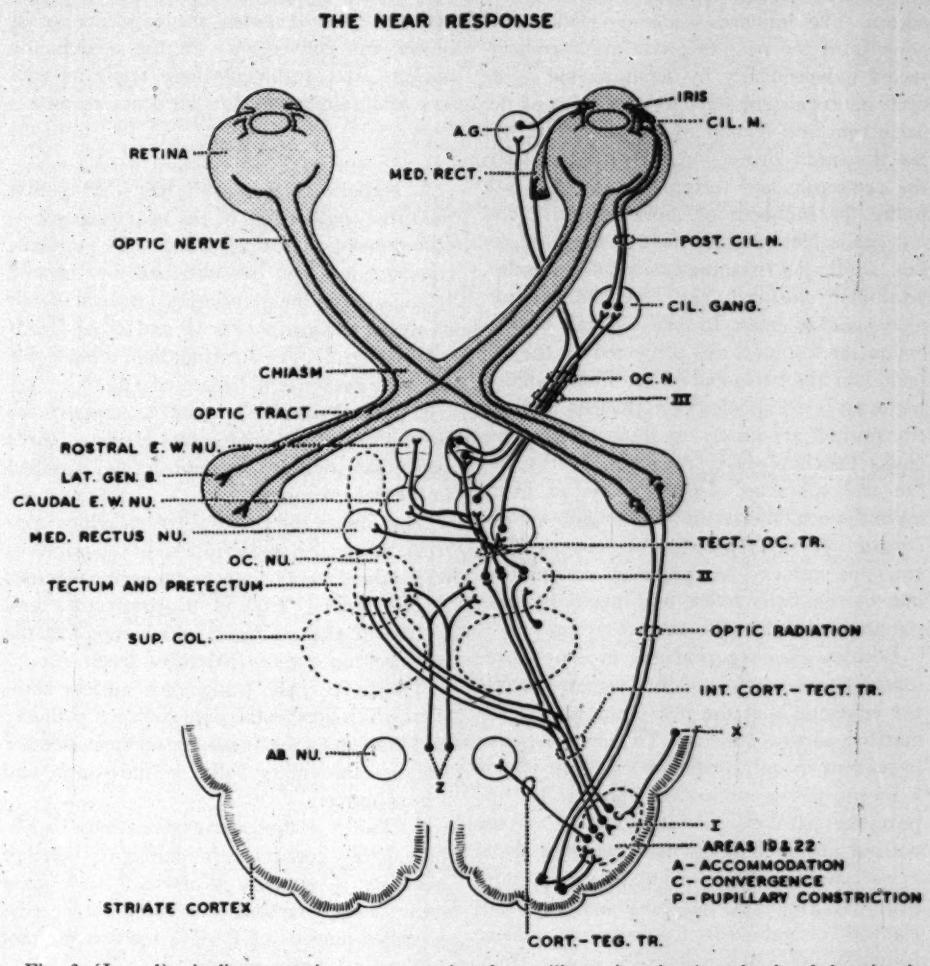

Fig. 3 (Jampel). A diagrammatic neuroanatomic scheme, illustrating the three levels of function in the nervous system involved in the near response (see text). (I) This indicates the functionally specific area for the near response in the preoccipital cortex. (II) This indicates the level of the tectum and pretectum. (III) This indicates the final common pathway for impulses to the ciliary muscle, medial rectus, and iris sphincter. (X) This represents the input of nervous activity from the entire cerebral cortex that modifies the output of the functionally specific areas for the near response in the preoccipital cortex. (Z) This represents the input of nervous activity from the spinal cord and brainstem which modifies the impulses discharged from the cerebral cortex into the tectum and pretectum. (A. G., accessory ganglion; AB. NU., abducens nucleus; CAUDAL E. W. NU., caudal Edinger Westphal nucleus; CIL. GANG., ciliary ganglion; CIL. M., ciliary muscle; CORT.-TEG. TR., cortico-tegmental tract; INT. CORT.-TECT. TR., internal cortico-tectal tract; LAT. GEN. B., lateral geniculate body; MED. RET., medial rectus; MED. RECTUS NU., medial rectus nucleus; OC. N., oculomotor nerve; OC. NU., oculomotor nucleus; POST. IL. N., posterior ciliary nerves; ROSTRAL E.W. NU., rostral Edinger Westphal nucleus; SUP. COL., superior colliculus; TECT.-OC. TR., tecto-oculomotor tract.)

modified by the total activity of the cerebral cortex. The impulses controlling the components of the near-response are then projected independently by separate, but adjacent, neuronal pathways to both sides of the pretectum and tectum of the midbrain, but the dominant projection is homolateral. In the pretectum and tectum the impulses fall under the influence of more primitive and less malleable systems, such as the light reflex, feedbacks from the extraocular muscles, vestibular, postural, and cerebellar mechanisms, and so forth. In their ultimate modification the impulses are projected on the cell bodies of the neurones of the final common pathway in the oculomotor nucleus and are transmitted separately to their effector organs—the ciliary body, the medial rectus, and the iris sphincter. Once the nerve fibers leave the oculomotor nucleus they do not decussate. There is probably a separate final common pathway for pupillary constriction due to the light reflex and pupillary constriction related to the near response.

Besides the experimental evidence presented above there are other cogent reasons for believing that the near response is primarily a cortical function. The near-response is present in only primates and man and is a recent phylogenetic development. Its appearance parallels the development of the cerebal cortex. It is the most complex visual motor phenomenon. Conjugate gaze, a more primitive function, has been assigned specialized cortical areas. Convergence, a more complex and phylogenetically newer function related intimately to the near-response, has been assigned a midbrain center. This is incongruous and contrary to neurologic thought. The idea of a midbrain center may be partially attributed to the difficulty with which convergence has been obtained by cortical stimulation.

Warwick[22] was presented morphologic evidence that disputes the existence of a center for convergence in the oculomotor nucleus. His arguments may apply as well to a midbrain center for the near-response.

Conclusion

1. Experimental work[25] has shown that the three components of the near-response—accommodation, convergence, and pupillary constriction—may be obtained by faradic stimulation of the preoccipital cerebral cortex of the monkey in Areas 19 and 22 of Brodmann (fig. 2). A neuroanatomic scheme for the near response is presented (fig. 3).

2. This evidence is used to support the proposition that there are at least three functional nervous system levels involved in the near-response:

The first is the cerebral cortex which controls, integrates, and synthesizes the impulses of the three components of the near-response.

The second level is the pretectum and tectum of the midbrain which modifies the impulses on a more primitive level.

The third is the oculomotor nuclear complex which acts as the final common pathway and transmits the impulses to their effector organs—the ciliary body, medial rectus, and iris sphincter.

3. The fact that convergence may be obtained by cortical stimulation is further evidence to support Warwick[22] that there is no integrating and controlling center (the so-called nucleus of Perlia) for convergence in the oculomotor nucleus.

450 Clarkson Avenue (3).

I wish to express my gratitude and indebtedness to Prof. Elizabeth Crosby of the University of Michigan for the use of the facilities of her department. Without her constant help and collaboration this work could never have been completed.

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Discussion

David G. Cogan, M.D. (Boston): Dr. Jampel has added substantially to the scanty experimental evidence for cortical representation of the near reflex. He finds that unilateral stimulation of an area in the peristriate cortex between temporal and occipital lobes will induce miosis, positive accommodation, and adductive movements in the monkey. That these adductive movements are associated with some measure of contraversive lateral gaze is perhaps not surprising and accounts, as the author states, for the apparent unilaterality or assymmetry in the convergent movements. It would be interesting to know if Dr. Jampel has tried bilateral and simultaneous stimulation of those cortical areas which he found to represent the near reflex.

The question in which we are all interested is, of course, the relevance of these observations to man. Specifically, what is the evidence based on analogous stimulation of the human brain and conversely what cerebral lesions have resulted in deficiency of the near reflex. Except for the equivocal paralysis of convergence with injuries to the occipital area I

know of no positive evidence for cortical representation of the near reflex in man although on inferential grounds it must exist and would presumably be situated in the visual association area. I personally have not recognized spasm of the near reflex as a manifestation of ictus, although I have seen it frequently as a functional disturbance. Nor do I recall that Penfield and Rasmussen (*The Cerebral Cortex of Man.* New York, Macmillan, 1950) noted convergence in their stimulation of the human cortex. However, it is entirely possible that the adversive movements of the eyes might have masked convergence and other aspects of the near reflex.

Dr. Jampel's interesting and significant studies on the monkey gives added impetus to document observations on the near reflex in human beings with disorders of the cerebrum.

Dr. Irene Loewenfeld (New York): Speaking for Dr. Lowenstein and myself, I would like to congratulate the speaker for a very beautiful, anatomically well-founded work.

We would like to make one additional remark on the question of our monkey. The problem which was dealt with in relation to this monkey was the problem of the Argyll Robertson syndrome. The hypothesis had been proposed that in the Argyll Robertson syndrome the iris itself is paralyzed and is merely dragged along by the ciliary body, and that therefore the pupillary near vision reflex survives in this syndrome while the light reflex does not survive.

The experiment of the monkey showed clearly that in the efferent third nerve the fibers for the light reflex and the fibers for the near vision contraction of the pupil are separate. From this fact nothing was concluded as to a nuclear versus a supranuclear integration of the oculomotor functions.

Dr. R. S. Jampel (closing): I think it would be an excellent idea to repeat those experiments in bilateral stimulation. We haven't done it.

As far as Penfield and Rasmussen were concerned, I think if you look at one ventral diagram in the most ventral portion of the cortex, they have an arrow that displays convergence movements, and that is the only instance of that in their experiments. I will recheck that when I have the opportunity.

As far as the convergence of the monkey is concerned, I think it is not difficult to test. It is much easier with the South American monkey, who will look at a raisin. His amplitudes of convergence are quite remarkable. The question arises as to whether the monkey really has a near response. This is something I investigated, not using the Rhesus monkey, who is a mean fellow, but the more placid South American monkey, who does converge and have marked pupillary constriction over quite large amplitude. The Rhesus is quite difficult to get to converge on a raisin.

It should be pointed out that a lot of these early experiments would bear repeating on the basis of the newer techniques and newer ideas. When you go through these papers you will immediately realize that. Maybe this will provide us with some very good neurophysiological evidence and will clear up our ideas on the origin of the strabismus, and so on, from the central nervous system point of view.

Dr. Loewenfeld did interpret our material in my own fashion. I did read that very excellent paper, and I did not bring up in the discussion the controversy or the evidence for the fact that there are two separate peripheral pathways for pupillary constriction, one governing near response and one governing the light response. If you read the paper of Foerster, Gagel and Mahoney, in which they extirpated the ciliary ganglion in the chimpanzee, in the particular animal the light reflection was eliminated but near response was intact. I think that ought to be work repeated, too. There are other things that would suggest that there are two separate pathways, but I did not go into it in this discussion. It is in my paper to a very small extent.

The important thing I want to emphasize is that, first, from your work, it is evident that these are carried independently. Dr. Lowenstein makes that point—that these are carried independently from the peripheral third nerve to the final peripheral organs. I think that is a significant fact, because it does indicate that there are discrete cells within the ocular motor nuclear complex that have to do with these functions. For some reason ophthalmologists have taken neurologic license and have made assumptions that the general neurologists would hesitate to make.

For example, they postulate that it must be two separate groups of cells governing the activities of the medial rectus. You won't find that postulation concerning any other muscle in the body—that there are two separate groups of cells for two different functions. The usual assumption is that there is a final common pathway, and that these enervational impulses to these bodies are varied by a supranuclear mechanism.

THE DARK-ADAPTATION PROCESS OF THE PUPILLOMOTOR PHOTORECEPTORS*

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INTRODUCTION

When a light is flashed into one eye the pupils of each eye constrict. Although it is evident that this is a direct consequence of a number of successive neurophysiological events the exact details are still far from certain. To begin at the very beginning, it is not known definitely whether the pupillomotor photoreceptors are: (a) exclusively rods, (b) exclusively cones, or (c) both rods and cones.

Evidence supporting all of the above possibilities is easy to find in the rather extensive literature on this question, which occupies a prominent place in the history of physiological optics. Since recent discussions of this problem[1,2] have already reviewed the literature in some detail it seems unnecessary to repeat an exhaustive literature summary, here also. Briefly, most attempts to answer this question have been devoted to studying: (a) pupillomotor responses to light stimuli in various parts of the visual field, (b) the photopupil response in totally color blind persons, (c) the pupillomotor threshold or (d) the Purkinje phenomenon, that is, the spectral sensitivity of the photopupil response at high and low light levels.

Interpretation of photopupillomotor perimetry is difficult because of the role played by light scattering within the media of the eye but a variety of evidence suggests that at least at high levels of illuminance the central part of the visual field is the more effective locus for a photic stimulus to evoke the pupil response[3-7] and this has usually been interpreted in terms of the "cones only" hypothesis.[3-6] Nevertheless if one uses monochromatic (green) light it has been demonstrated that the sensitivity of the photopupil response can be lower in the center of the field than in the periphery, in the dark adapted eye.[4]

The literature on the response of the pupil in monochromatism is confusing. Geldard[8] studied one such case and found ". . . only a very minute and immeasurable twitch . . ." of the pupil was evoked by light. Engelking[9,10] found in a similar case that in light adaptation the pupil was rigid (the German word is *starr*). On the other hand after dark adaptation the pupil responded with a "prompte Reaktion" to the first light stimulus equal in amount but somewhat slower than the response of the normal pupil in similar circumstances. Successive light stimuli, however, were progressively less effective as light adaptation progressed. Hess[11] found a prompt response of the pupil in a totally color blind eye in the dark adapted state which did not show any decrement after successive stimulation and moreover the response following light adaptation was also quite prevalent. One would be more concerned with these apparent contradictions if it were not now clear that there are several varieties of monochromatism.[12-17]

The determinations of the absolute threshold for the pupillomotor response are difficult to compare with psychophysical absolute thresholds because of the role of scattering and because there is every reason to believe that our instruments for detecting the minimum pupil changes are terribly crude when compared to the highly precise methods of modern psychophysics. Even so, comparisons have been made and authors have claimed that the photopupillomotor threshold is equal

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to that of the cones.[5,18] Other investigators have found thresholds for the photopupil response lower than the cone threshold but considerably larger than (about two logarithmic units above) the rod threshold.[1] If, however, the area of the test stimulus was gradually increased, the difference between the absolute visual threshold and the pupillomotor threshold became smaller and smaller and, when the test stimulus was a hemisphere covering the entire visual field, the two almost coincide.

Totally color-blind eyes have been reported to have a photopupillomotor spectral sensitivity curve with a λ_{max} in the green while the normal eye was said to show a λ_{max} in the yellow.[9] On the other hand Laurens[11] found in bright lights the pupil was smallest in (equal energy) light of wave length about 554.2 mμ while in dim light the smallest pupil was found with light of about 514 mμ. Wagman and Gulberg[20] tried to determine the minimum amount of light at various wave lengths through the spectrum required to produce a criterion pupil response. For minimum response criterion they found a spectral sensitivity curve which compared quite well with the rod luminosity curve but strangely enough this curve did not change at all as the response criterion increased! This latter finding when considered by itself suggests that "only the rods" are the responsible photoreceptors for pupillomotor response—an implication which neither Wagman and Gulberg nor anyone else has ever drawn.

In this paper the matter was studied by measuring the dark adaptation process of the pupillomotor photoreceptors—a question which until now has not been adequately investigated. A number of experimenters, however, have measured the size of the pupil at various time intervals in the dark following preadaptation to a rather bright light.[21-22] Brown and Page[2] in particular used this approach and actually compared the change in the size of the pupil in the dark to the change in threshold during dark adaptation. They found that the pupil size curve followed quite closely the dark adaptation curve of the central fovea and considered this as evidence that only the cones are responsible for eliciting the pupil response to light. In this connection it would seem important to know the magnitude of photopupillary response which can be evoked by a constant intensity light flash at various time intervals in the dark following constant light adaptation. If such information is available for a whole gamut of stimulus intensities, then the data can be used to decide the intensity of light which would be necessary at each time interval in the dark in order to evoke a given response criterion. It is *this* kind of a dark adaptation curve which would be most comparable to the psychophysical curves if one is interested in the nature of the pupillomotor photoreceptors. This is the procedure which has been followed in the present experiments.

Method

A. Adaptation

These measurements were made on two normal young adult males. The apparatus is illustrated in Figure 1. The subject was seated in a dark room and allowed to adapt to darkness for 900 seconds. Following this he allowed his left eye to light adapt for 600 seconds. For this purpose a piece of opal glass was fitted to cover the projector end of a 2×2 slide projector provided with a 300 w 120 v coil filament light source (S_3). The small tube was placed on the other side of the opal glass and the observer's left eye was placed in the open end of this tube with its entrance pupil about two inches from the opal glass. This provided light adaptation of $1.55\ (10)^4$ ft-L. to a region of the retina about 45 degrees in extent and symmetrically distributed about the line of sight.

Following the light adaptation period the observer placed his head into a previously adjusted chin and head rest and measurements were made of the response of the pupil to light of various intensities of stimula-

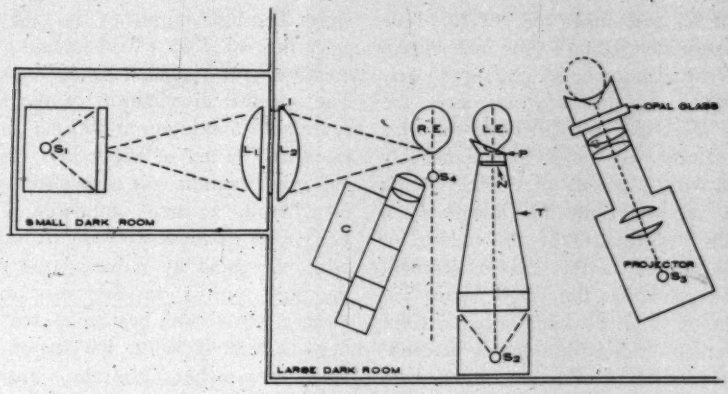

Fig. 1 (Alpern, et al.). Diagram of the apparatus.

tion at the following time intervals in the dark: 0.5, 1.0, 1.5, 2.0, 3.0, 4.5, 6.0, 8.0, 11.0, 16.0, 21.0, 30.0, 45.0, 60.0, and 80.0 minutes. In doing this two precautions were necessary in order that one could be certain that artefacts did not arise from the procedure.

In the first place it was necessary to be sure that the stimulus (test) flashes themselves did not interfere with the adaptation process. To be certain that this was the case 5.0-minute intervals were allowed to elapse between each stimulation by the test flash. Consequently it was not possible to take data for each time interval in the dark during each run, and the procedure had to be repeated a number of times with staggered stimulus presentations in order to obtain a response at each selected moment in the dark.

In the second place it was important to avoid so called "habituation" of the photopupil response. Recent neurophysiological studies[23–25] have shown that repeated stimulation with any one of a variety of sensory stimuli (among them both light and sound) is associated with a decrement in the amplitude of the physiological response to this stimulation. Loewenstein[26] almost 30 years before had found the same phenomenon in the photopupil response, which he later called "fatigue." To avoid this effect not only were at least five minutes allowed between successive stimulus presentations but only five stimulus presentations were ever permitted in any given experimental session.

In order to be certain that these precautions were adequate controls against such artefacts a number of measurements were made in the dark after 30, or 45, or 60, or 80 minutes without any preceding stimulus presentation at all during this particular dark adaptation interval. The responses so obtained did not differ from the measurements made in the usual way.

B. Infrared pupillography

Infrared pupillography has been demonstrated to be a valid method of studying the pupil response to photic stimulation by the pioneering work of Loewenstein (especially ref. 27) and by Talbot.[6] In the present experiments the modification of the infrared pupillographic apparatus proposed by Young and Biersdorff[28] was employed. For this purpose the helix (S_1) of a General Electric xenon flash tube (Ft 220) illuminated a ground glass which in turn was focused on the anterior segment of the subject's right eye by the lenses L_1 and L_2. The flash tube, ground glass and the lens L_1 were in a small dark chamber attached to the wall on the outside of the room in which the subject was sitting. A three-inch diameter circular hole was cut in the wall of the room

in front of L_2 and either side of this hole was completely covered by a four-inch square piece of Wratten number 88 (infrared) gelatin filter (I). In this way whenever the flash tube (S_1) was activated the anterior segment of the eye was instantaneously illuminated with a heavily concentrated area of invisible infrared light. The image of the pupil of the eye formed by the cornea together with the rest of the anterior segment was photographed on the High Speed Infrared film by a Grass kymograph oscilloscope camera (C) with film moving continuously at a speed of 50 mm./sec. The camera was started and the flash tube began flashing at a rate of 210 flashes/minute. Following the fourth infrared flash the stimulus (test) flash was presented to the left eye while pictures were made at a steady rate of the right eye. Pictures were taken until the response of the pupil was completed (upper limit of one half minute). The rate of flashing of S_1 was controlled by a General Radio Strobotac.

C. Stimulus and orientation light

The stimulus flash was provided by a second xenon flash tube (S_2) identical to that one used for photographing the right eye. This tube also illuminated a ground glass and the center of the ground glass was centered along the line of sight and 38 cm. from the center of the entrance pupil of the left eye. The ground glass and the discharge tube were mounted in a light tight casing by the manufacturer. A conical light tight cardboard tube (T) was attached to the other side of the ground glass and extended to the subject's left eye. At the eye end of this tube were mounted various densities (either 0, 1, 2, 3, 4, 4.5, or 5) of Wratten number 96 ("neutral" density) (N) filters, a 2.0 mm. in diameter artificial pupil (P) centered on the subject's line of sight, and a rubber eye shield to prevent any light leak to the photographed (that is, the opposite or right) eye. The test stimulus consisted of a circular flash of light approximately 21 degrees in diameter centered on the subject's line of sight. The light output of the tube showed a peak flux of 1.46 $(10)^6$ lumens; the flash duration was specified as 30 microseconds. The spectral distribution of the flash tube revealed a maximum at 490 mμ and a minimum at 750 mμ of about half the value at 490 mμ. Fixation was controlled by the filament from a small ophthalmoscope bulb (S_4) with its intensity reduced to near visibility threshold by reducing the current to the bulb with a variable resistance transformer. This bulb was mounted about 10 degrees directly below the line of sight and about two inches from the right eye. The subject used this to orient his gaze. Since it was too close to his eye to accommodate on, he saw only a blurred glow 10 degrees below his line of sight and he was instructed to stare off to infinity 10 degrees directly above this glow. After some practice both observers were able to do this satisfactorily.

Measurements were made on one subject (J.D.I.) for each intensity of stimulus flash and for the indicated times in the dark. For the second subject (H.D.) confirmatory data were obtained at only two different stimulus intensities (0.0 and 2.0 neutral density filters). For each stimulus intensity, observer, and time interval in the dark for which data were obtained measurements were made on three separate occasions.

The results were analyzed by projecting the photographic negative in a microfilm reader which increased the image of the pupil about ×13 its normal size. The vertical diameter of the pupil was measured in each case so that the obliquity of the optical axis of the camera in the horizontal (but not the vertical) plane could not alter the results.[29] To be certain that film shrinkage and slight shifts of the gaze did not change the magnification, the outer edges of the iris were measured also on each frame and this served to calibrate each picture. It was assumed that the absolute value of the outer diameter of the iris was equal to 10 mm. in calculating the size of the pupil.*

* This value was arbitrary and perhaps small by a mm. or two in each case. Since, however, the

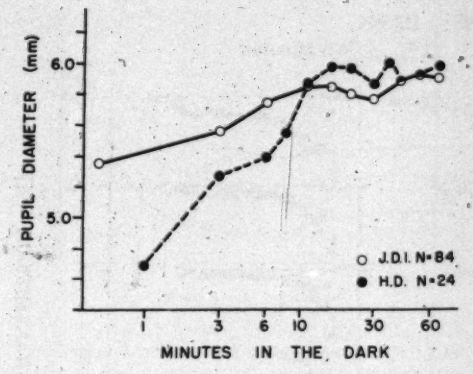

Fig. 2 (Alpern, et al.). Variation of the diameter of the pupil as a function of the duration in the dark following 600 seconds adaptation to 1.55 $(10)^4$ ft-L.

Results

A. Variation of the diameter of the pupil during dark adaptation

The data provided information concerning the size of the pupil in the dark following light adaptation. This analysis was made by averaging the measurements made of pupil diameter in the dark immediately prior to the presentation of the stimulus flash. The results of these measurements for the two observers are summarized in Figure 2 in which the abscissa axis shows the time interval in the dark (logarithmic scale) and the ordinate the diameter of the pupil. It is clear that for this intense amount of preadaptation the diameter of the pupil in the dark continues to increase (but only by relatively small amounts) long after the 10 minutes or so required for complete adaptation of the cones. These results are somewhat in conflict with those reported by Brown and Page[2] but the differences are undoubtedly related to the extremely intense amount of light adaptation employed in the present experiments as compared to the degree of light adaptation which they employed.

B. Analysis of the response

Selected responses from a single observer (J.D.I.) to various intensities of stimulus flash presented at various time intervals in the dark are illustrated in Figure 3. It is apparent even from gross inspection of these records that increasing the interval in the dark increases the response of the pupil to a stimulus flash just as increasing the intensity of the stimulus flash itself does. The question immediately arises as to how to estimate the magnitude of the response of the pupil to these instantaneous flashes. Most analysis of this kind quantify only the maximum change in the pupil diameter, or the maximum change in pupillary area. It is clear, however, from the data of Figure 3, that an important variable not included in such an analysis is the way in which the pupil changes size following these brief light pulses. In order to include this variable in the analysis we have reached the quite arbitrary decision of analyzing the response in terms of the integral of the pupil diameter vs. time curve. If P_o represents the diameter of the pupil in the dark and P its diameter at any moment (t) following the stimulus then the effect E of the flash can thus be operationally defined:

$$E = \int_0^\infty (P_o - P)dt. \quad (1.)$$

This definition of the effect of a flash proved quite satisfactory for these brief flashes although its use would probably have to be confined to flashes of light of duration less than the reaction time of the pupil (approximately 200 msec.). Since no really satisfactory relationship is available for integrating (1.) mathematically, the operation was carried out in each case with a planimeter.

C. Changes of the effect of a flash during dark adaptation

Figure 4 illustrates the data for the subject J.D.I. analyzed in this way. The figure

present study is concerned only with changes in pupil size relative to values found in the dark it did not seem necessary to determine this absolute value with any greater precision. This computation tends to make the absolute values for pupil diameter somewhat larger than those reported in these results.

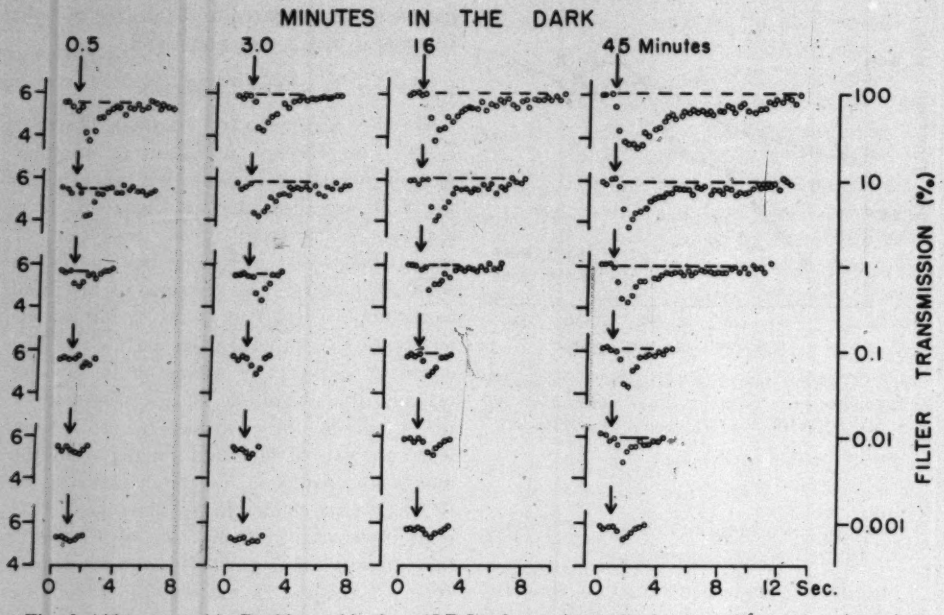

Fig. 3 (Alpern, et al.). Pupillographic data (J.D.I.) for various stimulus intensities and various time intervals in the dark. Responses were plotted only until the pupil returned to its initial size in the dark. Arrow indicates the moment at which the stimulus flash was presented. The ordinate of each graph is the diameter of the pupil in mm.

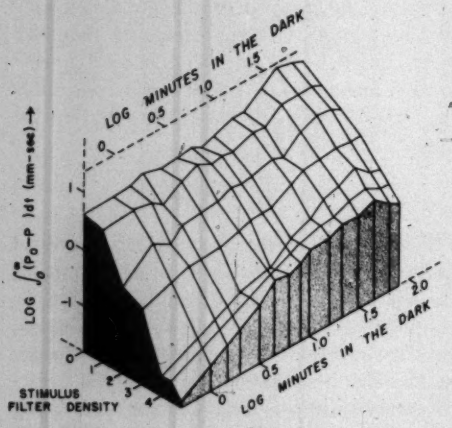

Fig. 4 (Alpern, et al.). Three dimensional logarithmic plot showing the responses produced by various intensities of stimulus lights and at various time intervals in the dark. The ordinates are values of the effect E of the stimulus flash as defined by (1.). Data are for subject J.D.I. and the plotted points are the means of three separate determinations.

is a triple logarithmic plot. The two horizontal axes represent respectively the density of the neutral filter before the left eye and the logarithm of the time interval in the dark following the light adaptation in minutes. The vertical axis represents the value of the logarithm of the effect of the stimulus flash (E) as defined by (1.). The greater the intensity of the stimulus flash the greater the effect of the flash at any given moment in the dark. Similarly prolonging the time interval in the dark with any given stimulus intensity is also associated with a greater and greater effect of the flash. However, it is quite clear that this latter process does not go on indefinitely. For the effect of a flash at any given stimulus intensity appears to reach a maximum after 45 minutes in the dark. For time intervals longer than this the stimulus flash actually has a slightly smaller effect.

The data in Figure 4 can be analyzed to

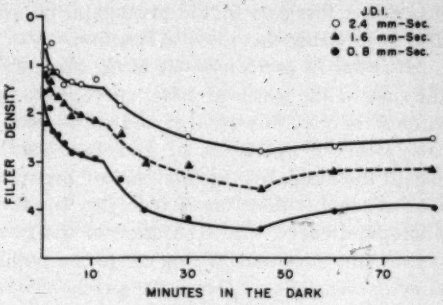

Fig. 5 (Alpern, et al.). Intensity of stimulus flash required to produce three different criteria of pupil response for subject J.D.I. Data are obtained by sectioning the three dimensional graph in Figure 4 at the indicated vertical levels.

decide the stimulus filter density required to produce any desired criterion response by making horizontal sections of this solid at any vertical level desired. The results of doing this at three different levels (2.4, 1.6, and 0.8 mm. sec.) is illustrated in Figure 5. In this figure the abscissa scale has been made linear, instead of logarithmic, in order to correspond more closely to the usual manner of presenting psychophysical dark adaptation curves. It is clear from Figure 5 that the pupillomotor "dark adaptation" curves have the same general shape irrespective of the criterion selected. It is easy to divide the curves into three separate parts:

a. There is a fast decrease in the intensity required to evoke a given response criterion for the first few minutes amounting from about 1.0 to 1.5 logarithmic units of stimulus intensity. This curve levels off after a few minutes and remains at about the same level until about eleven minutes in the dark.

b. There is a second sharp decrease in the intensity of the stimulus required to evoke a given response at about 10 minutes in the dark. This intensity value falls almost one and one half logarithmic units in the next half hour, first quite rapidly, later more slowly. It reaches a minimum after 45 minutes in the dark.

c. After 45 minutes in the dark there is a slow increase in the intensity of the stimulus required to evoke a criterion response. This rise is very small amounting to, at most, only one half logarithmic unit and it too seems to level off after 60 minutes in the dark.

Discussion

"Dark adaptation" curves like those illustrated in Figure 5 can now be compared to psychophysical dark adaptation curves. Measurements were made of the psychophysical dark adaptation curve under the same conditions of preadaptation. A modification of the McLaughlin dark adaptometer[30] was used to do this. Measurements were made with a 1.5-degree white test target in the temporal retina of the left eye, five degrees from the fixation point. The experiment was repeated on two separate days and the mean data are illustrated (as the open circles) in Figure 6. In this figure the ordinate is the logarithm of the threshold luminance of the test target in ft-L. For comparison the pupillomotor "dark adaptation" curve for the lowest criterion studied is placed on the same graph (solid circles) so that 0.001 ft-L of the psychophysical curve coincided with 3.0 filter density from the photopupil curve. The luminance units

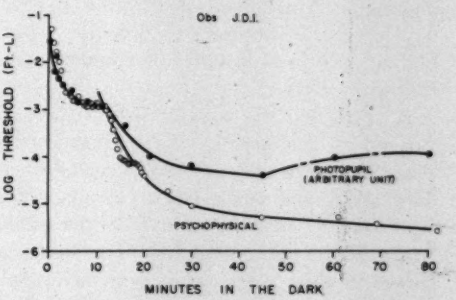

Fig. 6 (Alpern, et al.). Comparison of the psychophysical and photopupil dark adaptation curves. The lowest curve in Figure 5 is represented by the filled circles. The unfilled circles are psychophysical dark adaptation data for the same observer and the same preadaptation conditions. The ordinate scale is represented by the luminance of the psychophysical curve in foot lamberts (logarithmic scale) but the numbers also represent the filter density of the stimulus flash for the photopupil curve.

in the latter are, of course, arbitrary.*

It is clear from this analysis that remarkable agreement between the two sets of data exists for the first twelve minutes of dark adaptation. During this period both the pupillomotor and psychophysical curves drop a little over 1.5 logarithmic units and the forms of the two curves are almost identical—so much so that a single smooth curve can readily be drawn through both sets of data. The agreement between the sets of data for the remainder of the time in the dark is less striking than for the first 12 minutes, but similarities persist.

In the psychophysical curve the following 70 minutes in the dark are associated with a more or less smooth decrease in the threshold—the total decrement being about 2.5 logarithmic units. This drop is rapid up until 25 minutes in the dark and proceeds much more slowly from then on. The photopupil curve, also drops—rapidly up until 25 minutes then more slowly. But the rate of decrement even at its fastest is slower than the comparable part of the psychophysical curve and the maximum drop of this part of the curve is only 1.5 logarithmic units. This is achieved after only 45 minutes in the dark after which there is a slight increase in the photopupil "threshold" while the psychophysical curve continues to decrease. These findings are most readily interpreted in the following way:

A. *The first 12 minutes in the dark.* A variety of evidence has been mustered to show that this part of the psychophysical dark adaptation curve is due to adaptation of the cones[31] and the virtual identity of the forms of the photopupil and psychophysical curves in this region offers convincing evidence that this part of the photopupil curve also is probably due to cone function.

B. *From 12 to 45 minutes in the dark.* In the case of the psychophysical curve there is agreement that this part of the curve demonstrates the operation of rod function.[31] Again the similarity of this part of the two curves suggest that this is true also for the photopupil curve. The separation of the two curves, the fact that in this range the pupil curve never falls as far as the psychophysical curve at any given moment in the dark, would suggest that while the rods are operating to produce this curve the contribution that they make to photopupil response is considerably smaller than the cone contribution. That is what might be called the "photopupillomotor efficiency" of the rods is considerably less than that of the cones. Thus in Figure 6 the ratio of the drop in the pupil curve to the drop in the psychophysical curve is 0.834 for the "cone" curves but only 0.568 for the "rod" curves.

The pupil curve was determined with a 21 degrees centrally fixed stimulus while the psychophysical curve was made with a 1.5-degree target situated five degrees parafoveally. If these two targets were used in psychophysical experiments the two resulting cone curves would not differ very much from one another but the former target would give a rod curve considerably below the rod curve of the latter.[32] This would be due to the higher sensitivity of the peripheral retina[31,32] and the fact that while cones apparently summate quite inefficiently remarkably good spatial summation properties characterize rod vision.[33]

As a consequence the decline of the "rod" dark adaptation curve of the pupil at a much slower rate than the comparable part of the psychophysical curve in Figure 6 and the fact that it reaches a minimum value at least one logarithmic unit above the psychophysical curve at the same moment in the dark, suggest that the rods are even more inefficient pupillomotor photoreceptors than the above calculation indicates.

* In order to convert these units to those of the psychophysical curve, it would be necessary to integrate the time-light curve of the flash tube. The legitimacy of this procedure depends upon whether or not the intensity-time reciprocity law is valid for flashes as short as 30 microseconds. Since questions as to the validity of this law for flashes shorter than one millisecond have been raised recently, we have specified the luminance of the photopupil stimulus light only in arbitrary units.

This interpretation then, that both rods and cones are pupillomotor photoreceptors but that the rods are much less efficient in this respect than are the cones provides some clarification of the literature controversy already pointed out. Unless the eye is extremely dark adapted and scatter well controlled one would anticipate that a bright light in the center of the visual field would do a much more effective job in constricting the pupil (because of the regional distribution of photoreceptors) than would the same light in the peripheral field and this is what Talbot,[6] Harms,[5] Hess,[3] Alpern and Spencer,[7] among others have already found. Nonetheless stimulation of the rods under the proper condition can also bring about a pupil constriction even though the job it does is a relatively inefficient one. This is in keeping with the results found by Schweitzer[1] on threshold.

Flamant[34] studied pupil diameter as a function of luminance on 37 subjects under conditions of normal vision out of doors. She found that the data could not be represented by a single smooth sigmoid function as Reeves[21] had found. A discontinuity between two different smooth functions was present. The smooth function at low levels was responsible only for about 1.0 to 1.5 mm. change in pupil diameter and in terms of the above findings can be reasonably attributed to rods. At high levels the smooth function accounted for about 3.0 to 3.5 mm. of pupil diameter change, and this can quite properly be ascribed to cone vision. Between these two curves Flamant found a transition associated with twilight vision in which no change (or perhaps only a slight dilatation) occurred with increasing luminance.

The data on pupil size changes in total monochromatism are less easy to summarize and, as has already been indicated, at least part of the apparent contradictions in the literature must be due to the fact that a greater number of different types of monochromatism exist than most investigators have admitted.[35] If the interpretation in the present paper is correct, one would expect a small photopupil response in an eye completely devoid of cone function but with normal rod function. This is essentially what Geldard found in an observer* presumably of this kind.[8] The photopupil response for such an observer should have a rod spectral sensitivity curve and this is what Engelking[9] has reported although his colored stimuli were apparently not equated for equal energy.

Finally, if this interpretation is correct it is to be expected that the photopupil spectral sensitivity curve should demonstrate a Purkinje shift and Laurens[19] found this when he used pupil size as an ordinate for his spectral sensitivity curve. The failure of Wagman and Gulberg[20] to demonstrate anything but a rod spectral sensitivity curve, when the reciprocal of the intensity to produce a criterion pupil change was the ordinate, was probably due merely to an inability of these investigators to obtain monochromatic lights of sufficient intensity to evoke predominantly cone responses of the pupil.

C. *Forty-five to 80 minutes in the dark.* The psychophysical curve continues to fall while the photopupil curve suddenly rises before leveling off. The reason for this discrepancy is not clear. It is possible that this pupil effect could be due to some depression of the motor rather than sensory side of the response. For example, there might be a reduction of the responsivity of the iris muscles after prolonged period of relative dilatation in the dark. Such an explanation must await considerably more evidence for confirmation. Whatever its nature, there is little doubt as to the fact itself. The same effect was clearly evident in both observers and seems to be free of all probable instrument artefacts.

* It is by no means certain, however, that such observers have only rod vision! Detailed discussions of this problem by Walls and Heath[37] and by Sloan[36] lead to a contrary opinion but more recent evidence suggests that the earlier view[36] is probably the more correct one.[35]

Summary

The dark-adaption process of the pupillomotor photoreceptors was studied by determination of the stimulus intensity required to evoke a criterion pupil response at various time intervals in the dark. The curves so obtained show remarkable similarities (particularly in the so-called cone part) to psychophysical dark adaptation curves determined under identical preadaptation conditions. It seems evident that both rods and cones are capable of serving as pupillomotor photoreceptors but the latter are much more efficient in this capacity than the former.

University Hospital.

Discussion

Gerhard A. Brecher, M.D. (Atlanta): Looked upon from a general neurophysiological viewpoint the work of the authors concerns the problem of how nature relates information from one sensory input channel, the photoreceptors, to various motor output channels. Whereas the input information reaches sensory ganglion cells in cerebral cortical areas and elicits there sensory phenomena called light and color of varying subjective brightness, the strength of the output information to the motor areas must not necessarily parallel the intensity of the brightness sensations. The authors used the motor effects in one specific motor output area, the iris muscle, to test whether or not such parallelism exists. They thought that the degree of parallelism or the lack of it might reveal information about the details of the visual receptor mechanisms which are responsible for pupillary motor action.

Since it has been debated in the past, whether either the cones or the rods or both of them act as receptors for the pupillary reflex, the authors used the known information of the time course of the subjective dark adaptation curve as a tool of reasoning to differentiate between rods and cones as reflex initiating receptors. This avenue of approach is logical as it is based on the generally accepted concept that the break in the course of the dark adaptation curve is caused by the existence of two types of retinal receptors, the rods and cones, which have, according to v. Kries' duplicity theory, different response thresholds.

The reasoning of the authors was then that, if a parallelism of the classical dark adaptation curve and the threshold response curve of the pupillary reflex could be found, this would reveal the functioning of two distinct pupillary photoreceptors. These would probably be rods and cones. On the other hand if such parallelism could not be established the results would be inconclusive in this respect. The main task of a critical evaluation is to see whether the experimental design and execution was satisfactory to answer this question. It was.

One drawback of the method should, however, be pointed out. It is the impossibility to obtain one dark adaptation curve at one time since an interference of the test signal with the dark adaptation process must be avoided. The result of the investigation was the establishment of a pupillary response curve which parallels the subjective dark adaptation curve at the brake. In other respects the pupillary response curve turned out to be quite complex. It seems to resemble the curves obtained from electroretinographic studies. These findings were obtained from one subject only with supplementary data from a second one. This is a slim basis to draw conclusions from, considering especially that remarkable individual differences exist in the pupillary light reflex as pointed out again by Peterson (Acta Physiol. Scand., **37**: (Suppl.) 125, 1956). Obviously, data from additional subjects will be necessary to substantiate these studies. Nevertheless the authors are justified in arriving at their cardinal conclusion since here we are dealing with the rare case that one single good experiment with a positive result can answer a question. The conclusion of the authors that both the rods and the cones act as photoreceptors for the pupillomotor response is, therefore, valid. However, in my opinion, the data are insufficient to support any conclusions beyond this single one.

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NEURAL INTEGRATION AT THE RETINAL LEVEL AS EVIDENCED BY FLICKER FUSION MEASUREMENTS*

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INTRODUCTION

The use of flicker measurements to assess retinal function was proposed by us in 1958.[1] At that time the differences between our approach to the problem and those of previous and most current investigators was described. The apparatus developed and used since that report has been only slightly changed, and can be quickly described.

APPARATUS AND METHOD

The constant surround area is 50 degrees in diameter. In its center a small circular spot one degree in diameter is the flickering stimulus. This small area is alternated above and below the background at any controlled contrast from zero to 50 percent. The rate of alternation is accomplished by a rotating sector wheel driven by a synchronous motor fed with alternating current at any desired frequency between 30 and 63 cycles. This permits presentation of flicker rates from 22 to 46 cycles per second.

In the presently described experiments, the background was maintained at 60 cd/m^2, and the flicker area alternated between 57 and 63 cd/m^2, or at five percent above and five percent below the background.

Stimuli are presented in a constant order which appears random to the subject. Since differences were found between the rates of the right and left eyes, records for separate eyes are reported, rather than for subjects. When one eye is observing, the other is occluded with a translucent screen, preventing stimulation of the flicker to that eye, but maintaining its state of light adaptation.

Each change of rate is preceded by a warning signal. The subject responds by sounding, or failing to sound a buzzer. Every response is individually recorded. The period of flicker is about 10 seconds, so that from 200 to 450 stimulus changes are included in each trial.

These flicker stimuli were presented in two ranges, a slower range from 22 to 34 per second and an overlapping faster range from 30 to 46 per second. Eleven rates were presented in each range, or 22 per subject per eye, and each was repeated at "random" for a total of six times. Reported below are the records of over 38,000 such trials on 334 eyes.

Subjectively, it should be emphasized that the perception of flicker is quite different than the perception of alternating light. The perceptual phenomenon is quite recognizable as an entity, and appears as an erratic flashing of small spots within the flicker area, which become less frequent, and finally disappear completely when the flicker rate exceeds the critical fusion frequency.

TREATMENT OF DATA

For convenience in graphically presenting the data, as well as to better understand the time-intensity relationships which follow, the stimulus rate has been expressed in time of half cycles. That is, where the stimulus rate is 40 per second, 1/40th of a second represents a full cycle, and 1/80th the duration of each half cycle. Thus at 40 flickers per second, the light is brighter for 12.5 milliseconds, and dimmer for 12.5 milliseconds. The times of these half cycles therefore varied from 10.8 to 23 milliseconds throughout the experiment.

Perhaps the most important lesson physiologists can learn from modern atomic physics is the method physicists have used to deduce the characteristics of the many submolecular particles. These particles cannot be seen, nor measured, but their effects can be seen and measured. Similarly, we cannot see nor

* From the Eye Research Foundation. This study was done under contract Nonr-2750(00), between the Eye Research Foundation and the Office of Naval Research.

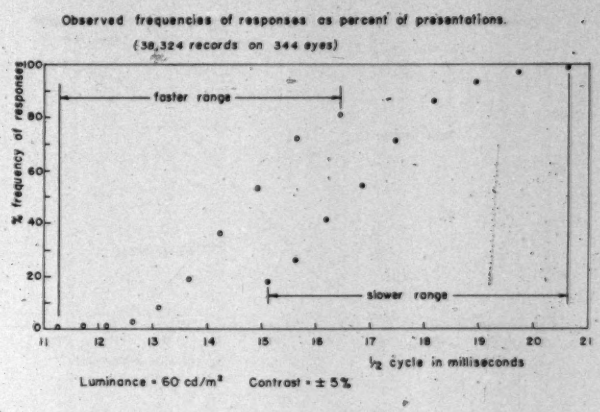

Fig. 1 (Peckham and Hart). Distributions of frequencies of responses of flickering stimuli. Open circles show faster range of stimuli, from 11 to 16.6 milliseconds (45 to 30 flickers per second). Solid circles show slower range of stimuli, from 15.2 to 22.7 milliseconds (33 to 22 flickers per second).

measure the intracellular activity of living nerve cells, but we can see and measure the effects. In order to do this, we use the tools of statistics. These tools may seem strange at first, because they are unfamiliar, but proper use of statistical tools can serve to describe neural behavior with new accuracy.

The statistical tools we shall use are concerned with measuring the variation of neural responses, as these neural responses are effective as perception. When a stimulus is presented to a subject, his response will be one of perception, or failure of perception. When the stimulus is strong, perception is nearly certain. When it is weak, perception may be absent, or very rare. Between these extremes, a given stimulus will not always arouse the perception, but will, as it starts from weak and becomes stronger, arouse perception with increasing probability. The technique of estimating the threshold of perception by determining the changes in probability of response is known as the psychometric method. It has been used for years, and is well established.

It is necessary to realize that both the stimulus and the responsive mechanisms are variable. However, each maintains its own peculiar variance, and therefore we can study the variability of behavior, in order to establish, by deductive logic, the nature of the neural responses which lead to perception.

In order to do this, it is essential that a large body of data be accumulated. From the study of the variance of these data, we may be able to establish the manner with which the human eye responds to certain specific light stimuli. Our method will be to examine the statistical appearance of the data, in probability arrays.

Description of results

An item analysis has been completed for all responses. This is plotted in Figure 1 as frequency of response against duration of one-half cycle. From its appearance, we can at once deduce that there are two probability curves, one for the slower range, and one for the faster range. That is, in the same subjects, a stimulus of 16 milliseconds per half cycle elicited "flicker" in 75 percent of the presentations when in the faster range, but only 34 percent in the slower range. The only difference between the two ranges is that speeds are different. There were no optical nor mechanical changes in the apparatus.

If we try to fit normal or Gaussian curves to the two sets separately, we get the results shown in Figure 2. The method of fitting the curves is that described by Finney.[2] The frequencies were plotted on "probability scale" graph paper, and the best fitting line was determined by the method of

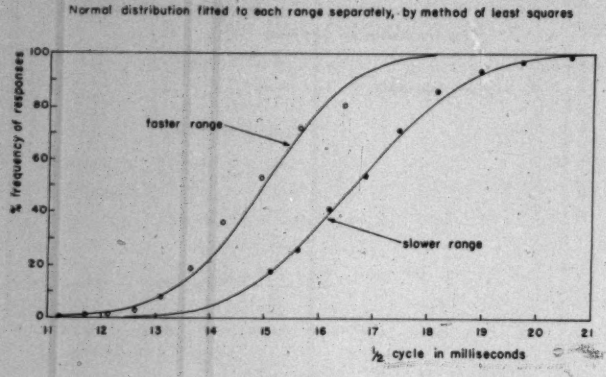

Fig. 2 (Peckham and Hart). Normal (Gaussian) distribution curves fitted to data of Figure 1. The fit is suggestive, but not adequate.

least squares. There are undoubtedly two distributions, but the data still do not fit well.

Blackwell has pointed out that threshold responses to visual stimuli should usually fit either "normal," "log-normal," or Poisson curves.[3] He correctly points out that determination of curve type is unlikely, unless the number of observations is very great, being in the order of more than 2,000 presentations. There are, here, sufficient numbers of data reported.

The "log-normal curve," is actually a transformed normal distribution. The function takes the mathematical form of replacing "log (X-A)" for "X" in the normal equation.[4] In our case, X represents the duration of the half cycle in milliseconds. The value for A must be determined empirically. It represents a limiting factor in the original distribution, which affects chance or probability of response as an independent constant. Unfortunately, Blackwell has limited his discussion to the special case, when $A = 0$.

As shown in Figure 3, when we plot the slower range as log (X-A) where $A = 0$, or as a simple logarithmic function, we find an excellent fit for the data. The faster range finds a satisfactory fit when $A = 11$.

Since these curves describe the data so nicely, we next consider the area between the curves. This is the shaded area in Figure 3.

Expressed as a distribution, in Figure 4, the difference area shows a normal bell-shape, slightly skewed. Plotted in its integrated form as a function of log (X-0) it

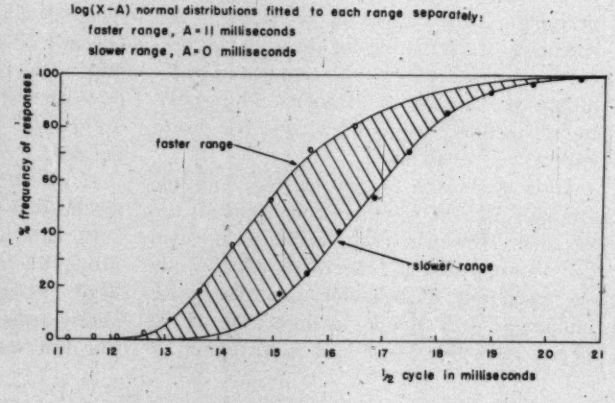

Fig. 3 (Peckham and Hart). Log-normal curves fitted to the data of Figure 1. The fit is excellent, when the logarithm factor takes the form of (X-A). For the slower range, A equals zero, that is, A is too small to estimate. For the faster range $A = 11 \pm 0.5$ milliseconds. The shaded area indicates the amount of enhancement, separately analyzed in Figure 4.

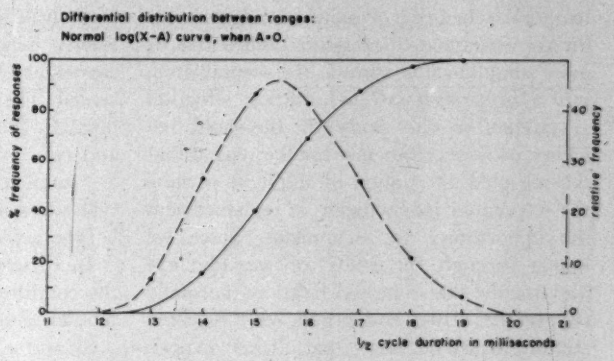

Fig. 4 (Peckham and Hart). The differential distribution between the two curves of Figure 3, shown as a relative frequency distribution (the broken line), and as a summated logarithmic Gaussian, where A = 0 (the solid line).

fits perfectly a log-normal distribution with zero limiting constant.

Discussion of results

Thus far our effort has been directed only towards describing the results. As part of this description it has been possible to express the results in the forms of clearly defined summated probability functions, the curves of which can be shown to nicely fit the actual distribution of the data. Now these curves have meaningfulness of themselves, and from this meaningfulness we wish to express our conception of the inferred theoretical behavior of the retina.

We have demonstrated three distinct functions, as evidenced by their statistical behavior patterns. The first function is the response to the slower range, which is a log-normal curve. In the factor (X-A), A equals zero. This function has a normal and typical response variance, and follows the Weber-Fechner law of linearly increased probability of perception as the logarithm of the stimulus time increases, other factors being held constant. We believe that this is the primary cone response in the macular area being examined.

A second function appears to be superimposed on the first, but within the same stimulus range. This second function requires a time factor in the expression (X-A). "A" must equal 11 milliseconds in order to obtain a proper probability fit for the distribution of the data. At 11 milliseconds duration, the term X-11 becomes zero, and the responses of both functions cease almost entirely. When X is about 15.5 milliseconds, X-A becomes 4.5 milliseconds, and the secondary function reaches its peak of enhancement over the primary function. When X becomes about 20 milliseconds, the primary function has reached certainty of response, and the secondary function is no longer effective.

Examination of the differential distribution, between the two curves, indicates a third function with a log-normal response curve, after the 11-millisecond time constant has been accounted for. This enhanced function follows the same type of Weber-Fechner law as the primary function, "A" equalling zero.

Theoretical implications

In the following discussion we must define our terms exactly. Stimuli are described with reference to the limen or threshold, which is the level of 50 percent probability of response. Supralimenal stimuli refer to stimuli of higher probability, that is, above 50 percent. Subliminal refers to stimuli of lower probability, that is, less than 50 percent. Enhancement refers to an increase in the measured probability of response of a given stimulus level. Summation refers to the accumulation of more than one stimulus into a single response. Summation can be

temporal, when two or more successive stimuli are accumulated, or spatial, when two or more simultaneous stimuli are accumulated, into a single response. The flicker situation is unique, in the study of threshold behavior of neural systems, for the reason that the repeated alternation of the light permits these peculiar phenomena of enhancement the opportunity to accumulate. Since we know, through the study of saccadic eye movements, that repeated fixations normally supply the visual end organ with repeated stimuli, it would seem that flicker experiments more closely approach the natural visual stimulus than do single flash experiments, and that, therefore, they represent a somewhat more adequate testing environment for the study of threshold phenomena.

It is interesting to turn to the functional description of retinal anatomy as presented by Polyak in further discussing our results.[5] Shown schematically in Figure 5, various elementary types of retinal neurons are presented. These include the cones, the primary bipolars (Polyak h-type), the ganglion cells, and two horizontal bipolar association cells, a "summating bipolar" (Polyak e- or f-type) and a "conditioning bipolar" (Polyak l- type association cell).

In Figure 5, at the left, we have indicated the condition in which only the direct cone-bipolar-ganglion neural channel is used (cones a, b and c). Unless the cone stimulus results in a signal capable of passing both the cone-bipolar and the bipolar-ganglion cell synapses, there will be no afferent signal, and no perception. Responses in the probability function shown in Figure 3 for the slower range could be accomplished by this simple and direct neural mechanism.

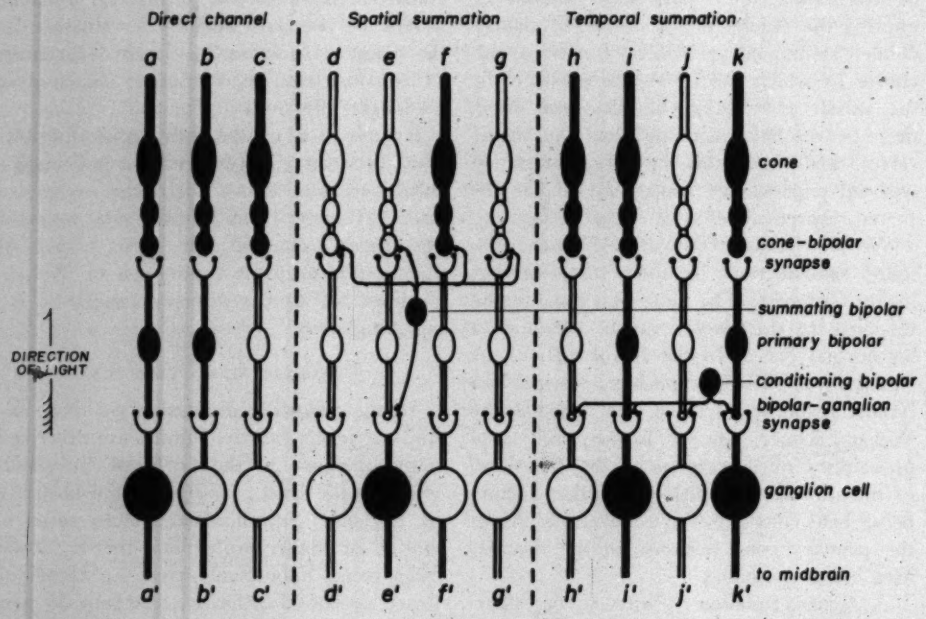

Fig. 5 (Peckham and Hart). Schematic representation of primary retinal synapses (modified from Polyak[5]). The cones, primary bipolars, ganglion cells, two types of summating bipolars, and synapses are indicated. The direct channels are shown as a-a′, b-b′, c-c′. Spatial summation is indicated in f-e′, through the mediation of a "summating bipolar." Temporal summation is indicated in i-i′, after mediation through a "conditioning bipolar," aroused by the firing through the channel k-k′. The data here presented contraindicate spatial summation, but support temporal summation.

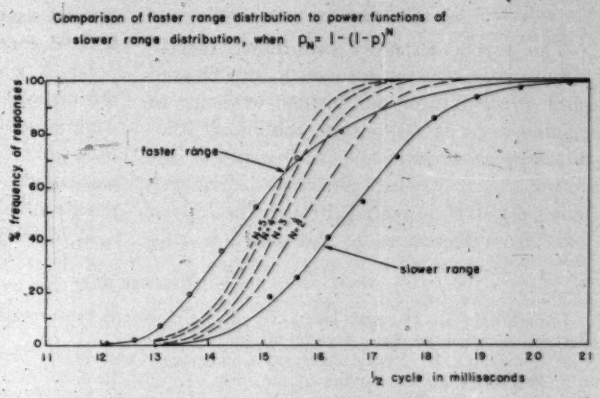

Fig. 6 (Peckham and Hart). Attempts to derive curves from the slower range to fit the data of the faster range by statistical expansion for spatial summations fail to yield a fit from slower range towards faster range.

Spatial summation of responses could be accrued by the inclusion of the summating bipolar shown in the center of Figure 5. Here, any cone which is stimulated may fire through a single summating bipolar cell (as f-e′). This increases the probability of an afferent signal, and a perceptual response. We can calculate the greater effectiveness of such a neural system as compared to the direct channel. Such a calculation requires the expression of new power functions of the basic slower range response, as shown in Figure 6. The calculation depends upon a factor "N" representing the number of cones summated. The slower range curve itself, represents N = 1, and increasing N to 2, 3, 4, or 5 fails to lead toward a semblance of fit for the faster range curve. Hence, as a mechanism to explain these particular data, the "summating bipolar" is inadequate. (Other stimulating conditions than those here reported might arouse this mechanism.)

A third schematic mechanism is shown in Figure 5 as temporal summation. The conditioning bipolar, when aroused to activity (say by the direct channel k-k′ to the right in the figure) would increase the probability of firing through the other bipolar-ganglion synapses. Any stimulus response, as at i, passing the cone-bipolar synapse is now almost certain to pass the bioplar-ganglion synapse. If the duration of such decreased synaptic resistance is about 11 milliseconds, *as under the specific conditions here reported,* the enhancement shown in the faster range curve could result. The form of this enhancement, corrected for the 11 millisecond interval, and impressed on a probability function, should find the log-normal distribution shown in Figure 4, since we have again only a simple neural chain involved in the response.

It is not difficult to appreciate why the enhancement is found in the faster range, and not the slower. In both ranges alternation rates are at random, but in the slower range the stimulus varies from supraliminal to liminal, and in the faster range, from subliminal to liminal. If the stimulus is supraliminal, all cells and all synapses will tend to discharge, with or without enhancing or conditioning mechanisms. With subliminal stimuli there will be fewer primary responses to the alternation, but those that do occur may serve to improve the receptivity of their neighbors. Hence we find that the faster range serves to elicit more responses than the slower, although the ranges overlap, and the extrapolated distributions are restrained within the same total range.

The data here reported refer to only one level of luminance, and to only one degree of contrast. Having discovered this new island, as it were, in the ocean of neurophysiological uncertainty, a fascinating program of exploration has been opened to us.

Summary

The psychometrically determined threshold of critical fusion frequency for low contrast foveal flicker has yielded evidence of enhancement of response to subliminal stimuli. Three mechanisms in the retina are considered to explain the results. The primary response is traced through the bipolar cells from the cones to the ganglion cells. Spatial summation, which could occur from several cones, through a special type of bipolar, to a single ganglion cell, is denied by the distribution form of the data. Temporal summation, within a specifically established time limit, is supported, as mediated through association cells operating at the bipolar-ganglion cell synapse level.

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Discussion

Dr. Francis H. Adler (Philadelphia): Drs. Peckham and Hart have drawn certain conclusions by subjecting their observed data to a mathematical treatment. Unfortunately, this mathematical treatment is far beyond my comprehension. However, my close friends will readily admit that this in no way reflects unfavorably upon the value of this paper.

There are certain things that seem to me worth inquiring about. To begin with, their conclusion that their data can best be explained by temporal summation rather than by spatial summation would seem to me to be true a priori, on the basis of the fact that their stimulus was a one-degree centrally fixated flickering light which varied in luminence against a constant background.

The fact that it was a one-degree centrally fixated stimulus means that the photoreceptors which were being examined were largely foveal cones, and of these a large porportion had a direct private-wire type of communication to the lateral geniculate body. Therefore, spatial summation would be hardly likely.

I wonder if they will repeat their measurements using a one-degree target situated, let us say, 20 degrees from fixation, which will include a population of photoreceptors of rods and cones having not a private-line type of communication but a party-line type of communication from which we would expect to find spatial summation.

Because of my obvious inadequacy to discuss this paper properly from the mathematical point of view, I called Dr. Johnson and suggested that Dr. Samuel Askovitz, of Philadelphia, he put on the program in my place. You all know of his mathematical ability. Unfortunately, the program had already gone to press, but he kindly accepted the invitation to come here and discuss it from the mathematical viewpoint.

Dr. Samuel Askovitz (Philadelphia): Thank you, Dr. Adler, for your kind remarks.

I have a number of comments on the authors' paper. First of all, I would like to commend them on the valuable improvement over their previous publication, namely, the fact that the average illumination of the central area was the same as the surround. Otherwise there is a problem of whether it is the difference in illumination that is being tested or whether it is really the flicker itself.

The authors are also to be commended on their use of a rather impressively large series of subjects and repeated trials. However, there are a few criticisms that I would like to bring up, more in the nature of inquiring whether the authors' explanation was intentionally made brief by limitations of time and space. Perhaps they can answer these questions to our complete satisfaction.

The question of conditioning by the range of speed of flicker that was used seems to me quite important. It was mentioned that at a speed of 16 milliseconds sometimes the response was 75-percent positive and sometimes the response was 34-percent positive. With this great a discrepancy in percentage of response, it seems to me it is difficult to imagine how any mathematical conclusions can be reached with any degree of accuracy.

If conditioning is of this great importance, then perhaps instead of a random presentation of speeds of flicker, some systematic presentation of the order of flicker should be made. For example, if the 16 millisecond speed was preceded, let us say, by 14, 15 or 17 milliseconds, these could conceivably still be completely within the rapid range or completely within the slower range. It was difficult to find why there was such a great difference.

The next question that I have to ask the authors is on the rather clear-cut distinction in the three possible types of response. What it seems to me their approach was, I would state as follows:

Either there is direct response, or there is spatial

summation, or there is temporal summation. It seems to me that sufficient consideration was not given to the possibility that there is a mixture of various types of response even in the same subject. Certainly with over 100 subjects perhaps some subjects responded in one way and others in a different way. In particular, in the use of N, the spatial summation of the number of impulses, perhaps some mixing of different values of N in the same response could give a different type of curve.

Also, I would like to point out that the types of curve used, namely, the logarithm of the normal Gaussian curve, and the reference made in the text to Poisson curves, are by no means the only types to fit this type of data. It is conceivable, for example, that an ordinary power series of Taylor-MacLaurin type could fit many types of data, or using trigonometrical sums, or various types of orthagonal polynomials.

The authors have done a fine piece of work in collecting this mass of data, but I would suggest that the data be presented in more detailed form so that others can make an independent analysis rather than rely upon the authors' methods as far as their mathematical techniques are concerned.

DR. R. H. PECKHAM (closing): I must say that it is most gratifying and pleasing to find people willing to take the time to read a paper, listen to a paper, and discuss it, and then to find them bringing up the same questions that we ask of ourselves.

If I may, I will go through the rough notes I made in the last few minutes and try to explain some of these questions, although not necessarily answer them.

Dr. Adler pointed out that had we done the experiment in an area of the retina in which there is spatial summation, we might have found spatial summation. This is true. Nevertheless, Polyak indicates the possibility of spatial summation in the cones and in the cone area of the fovea. Perhaps as many as eight or ten cones, under some circumstances, can be summated spatially as well as having "private wires" directly to the central nervous system through the lateral geniculates.

Consequently, although there is a possibility of spatial summation, the analysis of the data that we used (which may not be the right analysis) does indicate the inadequacy of a spatial summation.

Dr. Adler suggested 20 degrees from the point of fixation. Selig Hecht has shown that at six degrees from the point of fixation, which is a more comfortable fixating position, the rods and cones are equally effective, depending upon the brightness, and the typical dark adaptation curve with the cusp at about .001 footcandle represents the change from rods to cones at this retinal position. Data are being collected peripherally, but the present data refer only to the central area.

With regard to Dr. Askovitz' questions, which are even more fascinating to us because they represent the mathematical problem with which we were confronted, I thank him for his remark that we have improved our apparatus from our previous system, by alternating the luminance above and below that of the surround. We did it because we were interested in stimulating both above and below the level of adaptation, with low contrast.

Certain experiments that we will report, perhaps next year, will concern themselves with the differentiation as to whether the bright or the dark phase is the stimulus for the flicker response. This is a very important question, but we have no data to present with regard to its resolution.

The effect of the range of stimulus is a real puzzle. These were the same people, who saw 75 percent of the stimuli when presented in the rapid range, and 34 percent when the same stimuli were presented within the slower range. We actually did not set out to find the specific ranges that would be most effective. Had we performed this experiment under conditions in which the rate was almost entirely faster than the critical frequency, so that very few responses occurred, only a few of the subjects would have responded. The majority would not have responded at all. Conversely, had we chosen a slow range, high on the scale of response, nearly all the subjects would have responded to every stimulus.

In the analysis of the data, when we use the Probit Analysis method, values of response at zero and 100 percent can not be used. If we were to use a Poisson analysis it is possible that the rare responses could have been included in the analysis and hence that the analysis could have been made on the basis of rare responses. However, we chose this particular method because it permitted excellent fit for the data.

If you will refer to Figure 3, the slope of the curves is such that the standard deviation is approximately 4.0 milliseconds. The standard deviation for a single individual is only about 0.5 or 1.0 millisecond. In other words, very frequently an individual will respond to 42 cycles per second with no response, to 41 with nearly half responses, and to 40 with complete response, as an individual subject. The data represent the responses accumulated for all of the subjects, and consequently the standard deviation is spread out.

The peculiar phenomenon of enhancement that we found developed because we were fortunate enough to get within a centrally overlapping range. Had we changed the ranges, the appearance of the curves might have changed, but the conclusions would not have been affected.

We might use some other form for "N," this could be a function. The number of potential functions is infinite, and we run into the same problem by following Dr. Askovitz' suggestion of analyzing with respect to McLauren or Fourier functions. Although such other functions might fit, the function that we chose fits remarkably well. The analysis of the fitting of functions is beyond the scope of this paper, but by determining the X^2 probability of fit we find that the null hypothesis is in the order of 95 to 98 percent for the fit of these curves. This is a very, very close, excellent fit, and it would be doubtful that experimental data could be expected to fit any other form better.

THE CONSISTENCY OF THE 1955 CALIBRATION FOR VARIOUS TONOMETER WEIGHTS*

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As our efforts to improve the understanding and management of the glaucomas grow more purposeful and systematic, the reliability of the measurement of the various criteria that might lead to empirically and operationally defined categories becomes of paramount importance. Among these criteria, the intraocular pressure is of special importance since a large variety of clinical predictions is bsaed on it.

Furthermore, with the advent of tonography and the possibility of investigating intraocular fluid dynamics in the human, the reliability of the absolute value of the intraocular pressure has assumed added importance; for here the question must be answered whether a difference between findings reflects a biological difference or a technical one.[1]

In indentation (Schiøtz) tonometry, the estimate of intraocular pressure obtained from the calibration tables is adjusted for average values of two variables: the corneal curvature and the coefficient of ocular rigidity. This estimate is always "blurred" by the uncertainty of how much the values of a particular eye may deviate from the average values assumed in the calibration.

Friedenwald's ingenious work aimed at controlling these variables and obtaining a measurement of the intraocular pressure in the undisturbed eye by indentation tonometry is well known.[2-5] His experience with this system of measurement led him to conclude that the "error" of this system for the various weights is uniform and known, only for scale readings between 3.0 and 10 mm. Hg. This limitation makes it mandatory to use more than one weight in order to encompass the full range of pressures encountered clinically. Two weights are also needed to obtain a "qualitative" or a "quantitative" evaluation of the coefficient of rigidity in a particular eye. For this reason it is highly important to ascertain whether the calibration tables for the various weights are consistent; that is, whether for the same eye, the same estimate of the intraocular pressure will be obtained from the calibration tables irrespective of the weight used.

This question becomes even more significant when "the coefficient of ocular rigidity" is investigated as a biological variable that might be related to a clinical disease category.

These considerations made it desirable to evaluate the consistency of the corrected calibration tables of 1955. A clinical study was designed and conducted, for the past three years, under conditions which duplicated as nearly as possible, those under which the tables are supposed to be used.

GENERAL CONSIDERATIONS

In the design of this study, two approaches were considered:

1. To have tonometry with three weights performed on the same eye and repeated on various occasions.

2. To have tonometry with three weights performed only once.

The first approach would reduce the influence that "technical" and "clinical" errors of tonometry may have on the estimate of the "coefficient of ocular rigidity." However, the obvious disadvantage of this procedure is that repeated tonometry, unless restricted and spaced, alters the pressure.[4] On the other hand, measurements, repeated over long intervals, require the undesirable assumption that the coefficient of rigidity remains unchanged for a given eye.

Since the primary objective was not to

* From the Department of Ophthalmology, State University of Iowa. Presented at the meeting of the Midwestern Section of the Association for Research in Ophthalmology in Denver, Colorado, April 18, 1959.

obtain a more representative mean value, but rather to compare the performance of the various weights in measuring the pressure in the same eye, it was felt that the second approach was more preferable as a method of obtaining relevant and fruitful information.

Since in Schiøtz tonometry, the rate of outflow is increased because of the rise in the intraocular pressure from P_o to P_t, successive measurements should indicate a reduction in pressure if for no other than hydrodynamic reasons. Such a reduction does not, in itself, reflect an inconsistency in the calibration. However, as a variation, it may exaggerate or mask a possible "calibration" error, depending upon the direction of that error. Hence, it becomes necessary to include in the study a control which will help in evaluating the effect of repetition and, if needed, provide a correction factor for successive measurements.

Sources of variation

The variability of successive measurements may stem from technical, biological, or calibration sources. Adequate control, as well as standardization of the technical sources, makes the study more sensitive to the desirable variability that arises from the remaining two sources.

Technical sources of variation

In order to control and randomize this source of error, the following steps were taken regarding the measuring instrument and the method of performance of the test.

1. *The tonometer.* Mueller's electronic tonometers were used because of their greater sensitivity in recording plunger displacement. However, since the study required more than one tonometer, it became imperative to establish a reliable basis for comparing and selecting these tonometers.

One possible source of error is any deviation of the plunger weights from their nominal weight. Therefore, all plungers and plunger loads were weighed accurately with a balance sensitive to a tenth of a mg. All six tonometers tested proved satisfactory in this regard. The values related to the tonometer used in obtaining 75 percent of the data in this study appear in Table 1.

TABLE 1

Values of tonometer used

Nominal Weight (in gm.)	Actual Weight (in gm.)
5.5	5.4869 5.4871 5.4869
2.0	2.0016 2.0012 2.0011
4.5	4.4986 4.4988 4.4984

Further tests were concerned with the repeatability of the zero setting and the electronic performance of a tonometer against accurately measured mechanical displacements. A micrometer was designed with a precision ground test block and plunger to fit the nominal values of the tonometer footplate and plunger curvatures with an accuracy better than 1/10,000 of an inch. The micrometer, calibrated in 1/10,000 of an inch divisions, was mounted vertically to an aluminum frame with a support to hold the handle of the tonometer in place while its body rested vertically on the copper base. The plunger was perfectly aligned with that of the micrometer and moved freely with it. In this position, the tonometer was out of reach of the small magnetic field of the micrometer head (fig. 1-A).

With this device, it became possible to establish a constant zero point for all tonometers and test their electronic performance after the proper period of warm-up and calibration. Furthermore, it was possible to check the uniformity of the footplate and plunger curvatures of the tonometers and determine the nonlinearity of the scale reading between 0 and 10 by comparing the equivalent in scale readings of the precisely measured vertical displacement of the

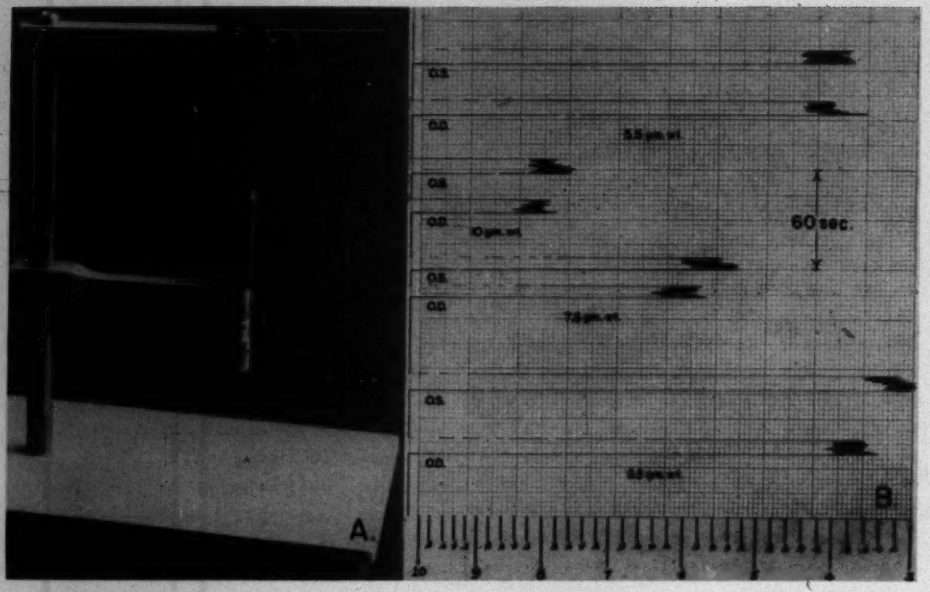

Fig. 1 (Armaly). (A) Calibrating micrometer. (B) Actual record of the procedure.

plunger with that recorded by the tonometer scale.

On the basis of these tests, four of the six tonometers were rejected for one or both of the following reasons:

a. The difference between the zero point obtained with the test block supplied with the tonometer and that obtained with the micrometer exceeded ± 0.2 scale units. Actually, differences as high as two units were found. Incidentally, similar differences were revealed when the zero point of one tonometer was determined with the test blocks of other tonometers.

b. The differences between the accurately measured equivalent of a certain scale reading on the micrometer and that recorded by the tonometer scale exceeded ± 0.2 units for a scale reading between 0 and 10.

The repeatability of a zero point was tested by removing the tonometer from the micrometer each time and mounting it again, repeating the determination several times. For the tonometers selected, repeated determinations were identical in the majority of trials and the difference never exceeded ± 0.2 of a scale unit.

To improve magnification of the final record, a step-down coupler was designed to connect the tonometer to a Leeds and Northrup recorder with which its sensitivity could be controlled and varied at will. A full scale deflection could be obtained for one scale reading with adequate stability. Since only scale readings between 3.0 and 10 mm. Hg were relevant to this study, the recorder was always calibrated to give a full scale deflection for the scale range of 3.0 to 10 mm. Hg.

The tonometer and recorder were connected to the main line through a voltage stabilizer.

The zero setting and calibration of the tonometer and recorder were checked on the micrometer before each test.

2. *The performance of the test.* A uniform procedure was adopted. With the patient in the recumbent position and the collar loose, the test was adequately and reassuringly explained. Anesthesia was attained by instil-

ling two drops of Proparacaine (Ophthaine) 0.5 percent on the cornea. Time was allowed for adequate anesthesia. The patient was then asked to fixate a 10-watt red bulb placed in the ceiling vertically above his head, and to maintain fixation with both eyes widely open.

The tonometer with the 5.5 gm. plunger was then held above and close to, but not touching, the cornea of the eye to be tested for a period of 10 to 15 seconds, then allowed to be supported completely and uniformly by the cornea for 10 to 15 seconds. The other eye was then tested in a similar manner. After this, the 2.0 gm. weight was added and the eyes tested again in the same sequence. The 2.0 gm. weight was then replaced by the 4.5 gm. weight, and the tests performed again. The 4.5 gm. weight was then removed and the tests repeated with the 5.5 gm. plunger. The time lapse between tests with various weights was that required to perform the change in plunger loads, and varied between five and 15 seconds (fig. 1-B).

Which eye was to be tested first was determined beforehand, and a record was made of the chosen sequence. "Squeezers" and patients unable to take the test without discomfort and without manual support of the lids were excluded.

Clinical and biological sources of variation

Since it is difficult to define, let alone control, the various possible patient factors that may influence this test, it was felt that randomization of these had the better chance of success.

For this reason a large sample was taken, including all ages between 15 and 95 years, and not unduly limited as to sex. None of those selected had any evidence of active or past sclerocorneal damage or intraocular disease other than errors of refraction or nuclear sclerosis. All had normal central visual fields.

Two hundred were selected from the refraction service of the State University of Iowa Eye Clinic without aiming at a random sample; in addition, 319 were recruited from a carefully selected sample that was representative of the general population of 40 years of age or older.

It may be stated here that the analysis of the results obtained from each sample showed differences in the absolute values of the measurements made with various weights, but identical results for the trends analyzed.

Since the objective of this study was not to investigate the distribution of pressure readings in the "normal" population, but rather to investigate differences in readings obtained with various weights on normal eyes, and since the analyzed trends were identical in both samples, the results of the analysis of the combined data will be presented. The ratio of females to males in the total sample was 4:3.

3. *Results and interpretation.* The means and standard deviations of the distribution of all values obtained with various weights appear in Table 2.

The first row refers to eyes on which it was possible to obtain scale readings between 3.0 and 10 mm. Hg with each of the three weights. The second row represents eyes on which such readings were obtained with the 5.5 and 7.5 gm. weights. The 10 gm. weight reading obtained was outside the 3.0 to 10 mm. Hg range and therefore, was discarded.

It is apparent that the means of the readings obtained on the same eye differ signifi-

TABLE 2

THE MEAN ± THE STANDARD DEVIATION OF PRESSURE READINGS WITH DIFFERENT WEIGHTS

$P^1_{5.5}$	$P_{7.5}$	$P_{10.0}$	$P^4_{5.5}$
19.21 ± 2.35	19.61 ± 2.39	20.10 ± 2.58	16.67 ± 3.70
17.32 ± 3.01	17.72 ± 3.02		15.92 ± 3.04
Ratio Factor			
0.19	0.46	0.80	

cantly from each other depending on the weight used. The estimate of the mean intraocular pressure obtained with the 7.5 gm. weight is larger than that obtained with the 5.5 gm. weight and smaller than that obtained with the 10 gm. weight. These differences between the means are statistically significant at the five-percent level of confidence for the 7.5 and 5.5 gm. weights, and at the 0.001-percent level of confidence for the 5.5 and 10.0 gm. weights.

It is to be noted that this difference is in a direction opposite that produced by repetition. The effect of repetition becomes apparent when the mean of the initial 5.5 gm.-weight reading is compared with that of the final 5.5 gm.-weight reading. Hence, repetition has partially masked the true discrepancy between the various weights; the true difference is larger.

The true value of the discrepancy was approximated by adding to the mean values obtained by each weight, that fraction of the total reduction from $P^1_{5.5}$ to $P^4_{5.5}$ that can be assigned to that step in the procedure. The total reduction depends on the rise in pressure from P_o to P_t in each measurement. It is also influenced by the facility of outflow, C, and the coefficient of ocular rigidity, K. The greater the P_t, C and K, the greater is the volume expressed and the greater is its effect on the intraocular pressure. Since it is safe to assume that the distribution of C and K in the sample is random,* the total reduction could be apportioned with respect to the mean P_t for each weight. This was done in proportion to the ratios of the weights,† considering that every reading is influenced by the weight used to determine it, as well as by the preceding weights. The share of each was then obtained by multiplying the total reduction by the ratio factors that appear in Table 2.

Another interesting difference is that between the mean total reduction of the first row (2.54) and that of the second row (1.40). All eyes had been treated alike, yet those in the first row were the ones with the higher initial pressure. This was interpreted as reflecting the nonlinear nature of the pressure-volume relationship and the fact that higher P_t values are obtained with higher P_o's, which lead to the expression of a larger volume of fluid from eyes that are more sensitive to volume loss because of the higher initial pressure.

In order to evaluate the validity of this approximation, 55 tests were performed using the 5.5 gm. weight to obtain all four determinations in otherwise the same routine as before. The means and standard deviations of the distribution of differences between successive readings appear in Table 3. Note that these differences gradually decrease with repetition because of the reduced sensitivity to loss of fluid at lower pressures. Yet all these differences are significant at the one-percent level of confidence.

The difference between the first and second reading is of great interest. It indicates that even one tonometry reduces the intraocular pressure significantly. In tonography the initial pressure is recorded on each eye first, then tonography is performed on the first and then on the second eye. This finding indicates that it should make a signifi-

TABLE 3

THE MEANS AND THE STANDARD DEVIATIONS OF THE DISTRIBUTIONS OF THE DIFFERENCE BETWEEN READINGS OF REPEATED TONOMETRY WITH 5.5 GM. WEIGHT PLUNGER

(The superscripts refer to the order of the reading)

	Mean	Standard Deviation
$P^1_{5.5}-P^2_{5.5}$	0.454	0.784
$P^2_{5.5}-P^3_{5.5}$	0.381	0.939
$P^3_{5.5}-P^4_{5.5}$	0.20	0.926
$P^1_{5.5}-P^3_{5.5}$	0.727	0.934
$P^1_{5.5}-P^4_{5.5}$	0.927	1.350

* Because of the absence of planned selection, and the improbability of accidental bias in a sample of this size.

† Using Mean P_t values yielded identical ratios.

cant difference in the calculated C values whether the P_o, appearing in the formula

$$C = \frac{\Delta V}{T \times (P_t \text{ av.} - P_o - \Delta P_v)}$$

is read from the scale reading at the beginning of the tonogram on the first eye, or from the tonometry. This difference makes the calculated C value smaller than the true value. Since the magnitude of this discrepancy is influenced by the facility of outflow, the under-estimation of C values will be greater in the normal than in the Glaucoma population. This may account for some of the undesirable overlap of the distributions of C values in these two groups.

In this regard it is of interest that if the mean total reduction ($P^1_{5.5} - P^4_{5.5}$) is multiplied by the ratio of the weights in Table 2 to those in Table 3, that is, $\frac{5.17}{4}$, the result 1.2 is not significantly different from the 1.4 recorded in Table 2. This provides strong support for the validity of the choice of the weight-ratios in apportioning the total reduction.

The correlation seen in Table 2 between the total reduction and the initial pressure, indicated an investigation of this correlation in the entire sample. The results appear in Figure 3 which show the mean reduction calculated for each pressure group. Note the high correlation between the magnitude of the reduction and that of the initial pressure reading. Differences between the magnitude of reduction in various pressure groups were statistically significant at the one-percent level.

When the total reduction was investigated in various age groups, another interesting correlation was found; the magnitude of the total reduction varied significantly in different age groups (fig. 2). However, in order to establish this correlation with age, it was important to rule out the possible difference in initial pressure between various age groups in the sample.

The means of $P^1_{5.5}$ calculated for each

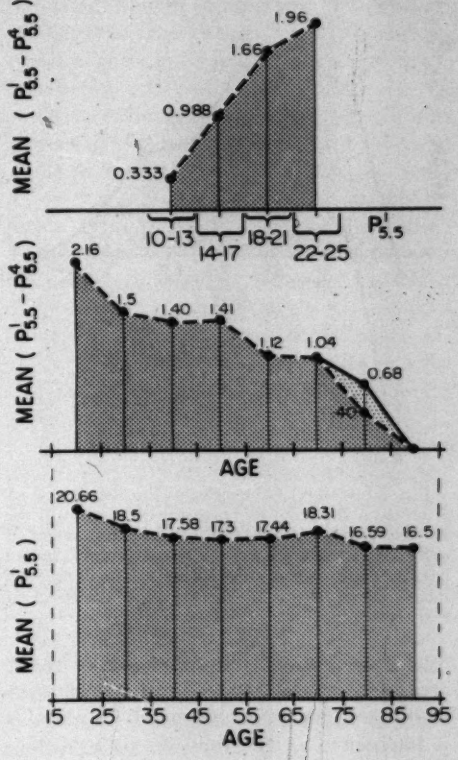

Fig. 2 (Armaly). The total reduction in relation to the initial pressure (upper graph) and age (middle graph). The mean total reduction for each designated pressure or age group is recorded. Dotted lines connect the means of uncorrected scores; solid lines connect the means corrected for the difference in initial pressure between the last two age groups and the remainder of the sample.

Lower graph: The mean of the distribution of pressure readings in each-age group. Except for the last two, all age groups had means that fell in one pressure group.

age group in the sample (fig. 3) did not fall in the same "pressure group." Therefore, it was necessary to correct those with lower pressure by utilizing the ratios obtained from the first graph of Figure 2. This correction was made for the age groups between 65 and 95 years and appears as a solid line in the second graph of Figure 2.

The correlation between total reduction and age is not so impressive as that between

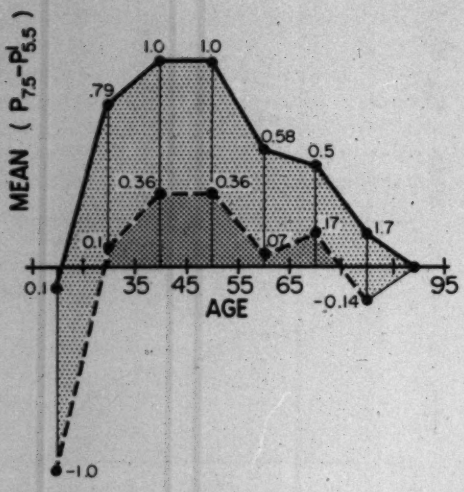

Fig. 3 (Armaly). The mean of the distribution of differences between the pressure readings of the 7.5 gm. plunger and that of the 5.5 gm. plunger in each eye, calculated for each age group. Dotted lines connect means of uncorrected scores; solid lines connect the means corrected for the effect of total reduction.

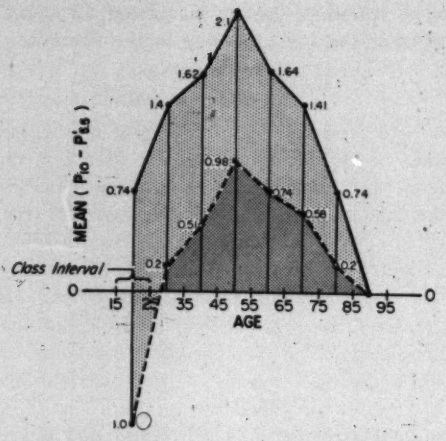

Fig. 4 (Armaly). Same as Figure 3 for differences between pressure readings of the 10 gm. plunger and those of the 5.5 gm. plunger.

total reduction and initial pressure. The decrease in total reduction with age becomes significant only after the age of 55 years. It is impossible to tell from this data whether this relationship reflects the effect of reduced facility, reduced rigidity, or a more general difference related to cardiovascular responses.

Since the total reduction correlates with pressure and age, it became important to investigate a possible correlation with sex in order to rule out the possible effect of the ratio of males to females in the sample. The total reduction is found to be identical for the two sexes, Table 4. Therefore, it is justifiable to speak of a correlation between total reduction and age.

Next, it is important to investigate how the difference between the reading of the 7.5 gm. plunger and those of the 5.5 gm. plunger correlates with age, initial pressure and sex, as well as the difference between the readings of the 10 gm. plunger and those of the 5.5 gm. plunger. The mean differences for each age group and initial pressure group are shown in Figures 3 and 4. The means of uncorrected scores appear in dotted lines, those corrected for the masking effect of reduction with repetition appear in solid lines.

The uncorrected as well as the corrected means were significantly different for different age groups. The distributions of $P_{7.5} - P'_{5.5}$ and that of $P_{10} - P'_{5.5}$, with respect to age were identical and parabolic and reach a maximum between the ages of 40 and 50 years. This discrete and significant correlation between the differences among readings with various weights and age cannot be regarded only as a manifestation of the calibration error. Instead, it should be looked

TABLE 4

THE MEANS AND THE STANDARD DEVIATIONS OF THE DISTRIBUTIONS OF DIFFERENCES BETWEEN READINGS WITH VARIOUS WEIGHTS OBTAINED IN THE TWO SEXES

(The superscripts refer to the order of the reading)

	Males Mean ± S.D.	Females Mean ± S.D.
$P_{7.5} - P'_{5.5}$	−0.008 ± 1.21	0.48 ± 1.20
$P_{10.5} - P'_{5.5}$	0.47 ± 1.30	1.60 ± 1.40
$P'_{5.5} - P^{4}_{5.5}$	1.42 ± 2.20	1.32 ± 2.15

upon as representing the distribution of a biological variable that can influence the difference between readings with various weights. The most likely factor is the coefficient of ocular rigidity. These curves then represent the variation of ocular rigidity with age.

The distributions of these differences with respect to initial pressure are shown in Figure 5. The means of the uncorrected scores shown in dotted lines, vary inversely and significantly with the initial pressure. However, before one can interpret this as a relationship between rigidity and initial pressure, or between the true discrepancy and initial pressure, it is necessary to show that this relationship is independent of repetition.

When the effect of repetition is corrected for, the mean differences of the three weights are no longer significantly different for pressure readings between 14 and 25 mm. Hg; yet, despite this correction, these differences remained significantly less for pressure readings between 10 and 13 mm. Hg for the 5.5 and 7.5 gm. plungers (see solid lines in Figure 5). This means that the discrepancy or error in the calibration of the three weights is uniform for pressure readings between 14 and 25 mm. Hg and becomes significantly less for pressure readings between 10 and 13 mm. Hg.

These differences were investigated with respect to sex, and the results are shown in Table 4. The mean difference in the males differed significantly from that in the females. Considering that the total reduction was identical for the two sexes, it becomes most unlikely that this difference reflects a difference in the reaction of the two sexes to tonometry, but a difference in the ocular rigidity of the two sexes. Hence, the coefficient of ocular rigidity is higher in the females.

The relation of the total reduction to the uncorrected difference between readings obtained with various weights was investigated for each eye. The results are shown in the upper tracings of Figures 6 and 7, which indicate that as the difference becomes positive and greater the mean total reduction becomes less. However, for this to be interpreted as representing the relationship between the effect of repetition and ocular rigidity, it is important to show that it is not caused by the procedure.

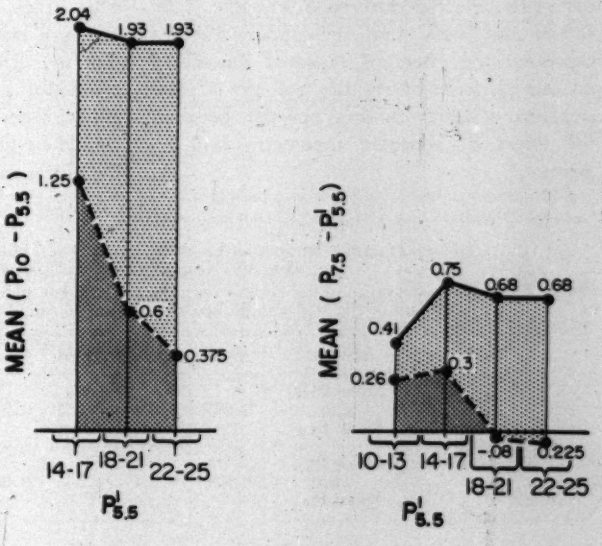

Fig. 5 (Armaly). The mean of the distribution of the difference between readings of various weights, investigated for different initial pressure groups.

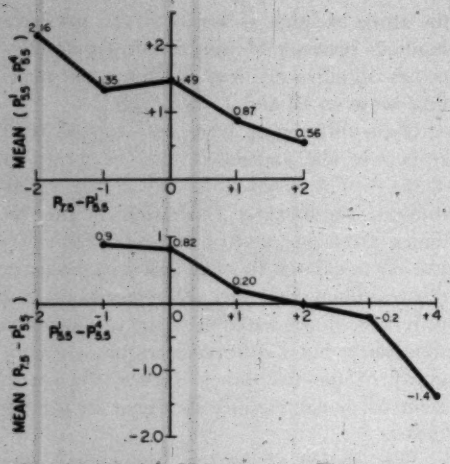

Fig. 6 (Armaly). Upper tracing: The mean total reduction investigated with respect to the difference between readings of various weights.

Lower tracing: The mean of the distribution of the differences between readings of various weights investigated with respect to magnitude of total reduction.

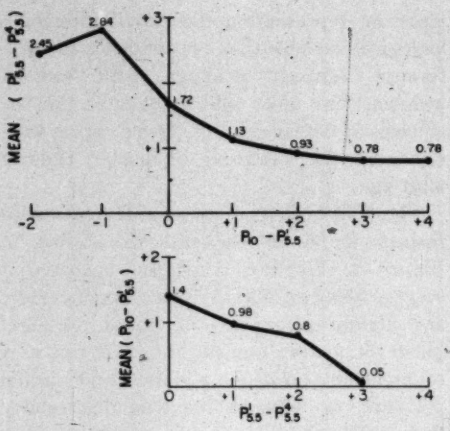

Fig. 7 (Armaly). The differences between readings of the 10 gm. and 5.5 gm. plungers treated in a manner similar to Figure 6 and with respect to total reduction.

For this purpose, the uncorrected difference was investigated for various magnitudes of total reduction and the means are shown in the lower tracings of Figures 6 and 7. This relationship indicated that when the total reduction was greater the uncorrected or detected difference was less. This favors our view that the upper tracings represent the effect of repeated tonometry on our estimate of rigidity and not a relationship, contrary to the expected, between the effect of repeated tonometry and rigidity.

This demonstrates that the estimate of rigidity in any one eye depends on the inevitable effect of repeated tonometry, even when this procedure is performed only once. Repetition of tonometry with two weights does not reduce this dependency.

These findings also indicate that we always underestimate the coefficient of ocular rigidity because of the effect of repetition. This error is greater in eyes with higher pressure because the reduction with repetition in such eyes is greater. Therefore, in evaluating methods of measuring ocular rigidity, other factors being equal, preference should be given to those which least disturb the intraocular pressure.

TABLE 5

THE COEFFICIENT OF OCULAR RIGIDITY, CALCULATED FOR EACH AGE GROUP FROM THE 1955 NOMOGRAM OF FRIEDENWALD

(In each age group the mean initial pressure reading with 5.5 gm. plunger and the corrected mean reading of the 7.5 and of the 10.0 gm. plungers were used to calculate the rigidity coefficient)

	Age (yr.)	15–24	25–34	35–44	45–54	55–64	65–74	75–84	85–94
Coefficient of Rigidity Based on	Mean $P'_{5.5}$ and Mean $P_{7.5}$	0.020	0.030	0.035	0.040	0.045	0.040	0.028	0.022
	Mean $P'_{5.5}$ and Mean $P_{10.0}$	0.028	0.030	0.035	0.040	0.035	0.035	0.028	0.022

The coefficient of ocular rigidity for various age groups was calculated from Friedenwald's 1955 nomogram, using the mean corrected scores of $P_{7.5} - P^1_{5.5}$ and the mean $P^1_{5.5}$, and similarly with the $P_{10} - P^1_{5.5}$. The values are shown in Table 5. They also demonstrate that this coefficient does not increase linearly with age but has a parabolic distribution.

Summary

The procedure and results of a clinical investigation of the consistency of the 1955 calibration for various weights of the Schiøtz tonometer are reported. The sample consisted of 500 subjects with normal eyes.

The characteristics of the footplate and plunger were found to vary markedly in the six Mueller's electronic tonometers investigated. This nonuniformity produced significant differences between measurements obtained with different tonometers.

The results reveal that a significant discrepancy exists in the calibration of the 5.5, 7.5 and 10 gm. plunger weights. Larger weights yield higher estimates of the intraocular pressure. This inconsistency is uniform for pressure readings between 14 and 25 mm. Hg, but is significantly lower for readings between 10 and 15 mm. Hg.

The difference between readings obtained with various weights on the same eye was found to correlate with age and sex. The distribution of the coefficient of ocular rigidity in various age groups was not linear but parabolic and reached a maximum at 40 to 55 years of age. It was significantly greater in women than in men. The position of maximum raises the question of whether ocular rigidity is intimately related to the development of certain disease categories of this age group, especially the "glaucomas." So far, ocular rigidity has been considered as a source of uncertainty in measuring the intraocular pressure with indentation tonometry. These findings suggest that improved techniques of measurement of this coefficient may uncover important physiopathologic attributes.

Single tonometry produced a significant reduction in intraocular pressure which can be accounted for by massage. This effect tends to increase the overlap of tonographic estimates in the normal and glaucoma population. It also leads to an underestimation of ocular rigidity, especially in eyes with higher initial pressure.

Confidence in the calibration based on clinical studies should be guarded and evaluated with great vigilance. Such studies introduce the effect of various biological variables which are in themselves capable of producing significant differences in the results of tonometry.

University Hospitals.

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ASSOCIATION FOR RESEARCH IN OPHTHALMOLOGY, INC.

BUSINESS SESSION

Tuesday, June 9, 1959

CHAIRMAN ROMAINE: At this time we will take a short period for a business meeting.

I would like to say a few words about the association. I think any association that has grown from a membership of less than 50 to almost 1,100 members deserves some recognition. Thanks ought to go to the people who organized this association and brought it through its growing pains.

I would like to mention some of our "greats": Dr. Berens, Dr. Heath, Dr. Woods, Dr. Benedict, the Drs. Post, Dr. Bedell, Dr. Vail, Dr. Harley, and many others who put their shoulders to the wheel and gave this organization a start. Also, the people who kept it going and progressing as it should receive our thanks.

The organization of the sections and the election of the section officers have, I think, enhanced the work of the national organization. I believe this year the fact that many of the sections are having two- and three-day programs indicates a tremendous increase in progress.

I would like to thank Dr. Vail and Mrs. Chalkley for the help they have given us in publication in THE AMERICAN JOURNAL. I believe this has helped to enhance the strength of the organization. The scientific centers have also increased their facilities and made it possible for better work to be done.

Last, but certainly not least, the tremendous administrative job done by Dr. Johnson and Miss Warren in building up the number of members of the association, and increasing their interest in it, is deeply appreciated.

I would like to thank the local arrangements committee, Dr. Haigh and all those who have helped him in making this meeting possible and the members of the Section on Ophthalmology of the American Medical Association for their help and co-operation, and most certainly the essayists who are contributing their time and good work.

I would like to say a word about the first midwinter meeting that was held last year, and to thank Dr. Allen for making it possible. I think it is a big asset to the association to have a second national meeting. The thought at this time is to combine this second national meeting with one of the sectional meetings. It will be held this year with the Southeastern Section, December 3rd, 4th, and 5th at Augusta, Georgia, under the chairmanship of Dr. Fair. The trustees feel that a combined meeting will help the Section, as well as the midwinter meeting. Perhaps the term "midwinter" meeting in December is a little farfetched but this seems to be a convenient time for the meeting.

We come next to committee reports. In the absence of Dr. McDonald, Dr. Fair has been so kind as to say he will give the report of the nominating committee.

DR. FAIR: Mr. Chairman, I should like to nominate Dr. Irving Leopold for the position of trustee, and Dr. Lorand Johnson as secretary-treasurer.

CHAIRMAN ROMAINE: Dr. Irving Leopold is nominated as trustee and Dr. Lorand Johnson as secretary-treasurer. Are there any other nominations? If not, it is the usual method to have one vote cast, so I will ask Dr. Fair if he will cast one vote for the slate.

DR. FAIR: I cast the vote, Mr. Chairman.

CHAIRMAN ROMAINE: Also, since he is in his working clothes, I will ask Dr. Fair to substitute for Dr. Chamberlain, who apparently is not here, and Dr. Henry Allen, who constitute the auditing committee.

DR. FAIR: I am told that the report of the certified public accountant has been inspected by Dr. Chamberlain and Dr. Allen and approved by them.

CHAIRMAN ROMAINE: Thank you, Dr. Fair.

The Board of Trustees has suggested the following people for honorary memberships: Rose Alexander, Mary Latzker, John Dunnington, and Frederick Cordes. If this meets with the approval of the membership, we will consider them accepted. They are accepted.

The last report is that of our very good secretary-treasurer, Dr. Johnson.

SECRETARY LORAND JOHNSON: The auditor's report has been approved by the auditing committee and printed in the Spring issue of the Supplement. I regret this Supplement was not ready prior to the meeting, but you will receive it accompanying the July issue of THE AMERICAN JOURNAL OF OPHTHALMOLOGY.

I may report that the cash in the bank as of January 1, 1958 was $11,480. There were 109 new members voted into membership yesterday. The total paid membership as of June 1, 1959 is 1,097. Thank you.

CHAIRMAN ROMAINE: Thank you, Dr. Johnson. We will have a 10-minute intermission. I don't believe it will give anyone time enough to go swimming, so don't try it.